UNITED STATES ENVIRONMENTAL PROTECTION AGENCY
WASHINGTON, D.C. 20460

OFFICE OF
PREVENTION, PESTICIDES AND
TOXIC SUBSTANCES

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MEMORANDUM

DATE: June 5, 2007

SUBJECT: ***para*-DICHLOROBENZENE:** Third Report of the Cancer Assessment Review Committee

PC Code: 061501

FROM: Jessica Kidwell, Executive Secretary
Cancer Assessment Review Committee
Health Effects Division (7509P)

TO: George Ghali, Toxicologist (TB)
Nancy McCarroll, Toxicologist (TB)
William Donovan, Risk Assessor (RRB3)
Health Effects Division (7509P)

Molly Clayton, Chemical Review Manager
Reregistration Branch 2
Special Review & Reregistration Division (7508P)

The Cancer Assessment Review Committee met on February 21, 2007 to re-evaluate the carcinogenic potential of *para*-Dichlorobezene. Attached please find the Final Cancer Assessment Document.

cc: J. Fletcher
Y. Woo

CANCER ASSESSMENT DOCUMENT

THIRD EVALUATION OF THE CARCINOGENIC POTENTIAL OF

para-DICHLOROBENZENE
(also known as 1,4-DICHLOROBENZENE)

PC CODE 061501

FINAL
June 5, 2007

CANCER ASSESSMENT REVIEW COMMITTEE
HEALTH EFFECTS DIVISION
OFFICE OF PESTICIDE PROGRAMS

DATA PRESENTATION: George Ghali, Toxicologist

DOCUMENT PREPARATION: Jessica Kidwell, Executive Secretary

COMMITTEE MEMBERS IN ATTENDANCE: (Signature indicates concurrence with the assessment unless otherwise noted.)

Gregory Akerman

Karlyn Bailey

Lori Brunsman, Statistician

William Burnam, Chair

Marion Copley

Ray Kent

Mary Manibusan for MM

Nancy McCarroll

Robert Mitkus

Jess Rowland

NON-COMMITTEE MEMBERS IN ATTENDANCE: (Signature indicates concurrence with the pathology report)

John Pletcher, Consulting Pathologist

OTHER ATTENDEES: William Donovan (HED/RRB3), Gino Scarano (HED/TB), Kevin Costello (SRRD)

TABLE OF CONTENTS

EXECUTIVE SUMMARY

On February 21, 2007, the Cancer Assessment Review Committee (CARC) of the Health Effects Division (HED) of the Office of Pesticide Programs (OPP) met to re-evaluate the carcinogenic potential of *para*-Dichlorobenzene (*p*-DCB) (also known as 1,4-dichlorobenzene). This was the third cancer evaluation for this chemical. *para*-Dichlorobenzene was previously classified in 1989 as a Category C carcinogen. The purpose of this 2007 meeting was to evaluate new inhalation data (an inhalation study in rats and mice by Aiso et al 2005a) and to determine the appropriate cancer classification of this chemical. The status of the rat and mouse tumors that were examined in the previous cancer assessments, and which are not under reexamination in this CARC assessment, was summarized and discussed as part of the weight-of-the-evidence determination of the cancer classification.

Dr. George Ghali of the Toxicology Branch presented background information on this chemical, the conclusions of the previous cancer meetings, and the new inhalation chronic toxicity and carcinogenicity studies in mice and rats by Aiso et al. 2005a. Aiso et al., 2005a have published an article summarizing the findings of carcinogenicity studies in F344 rats and BDF1 mice exposed via inhalation to *p*-DCB, which is the focus of this document. In this study, groups of 50 F344 male and 50 female rats or 50 BDF1 male and 50 female mice were exposed to vapor of *p*-DCB at concentration levels of 0, 20, 75 or 300 ppm *p*-DCB via whole body inhalation for 6 hours/day, 5 days/week for 2 years. Dr. Ghali also presented the structural activity (SAR) and metabolism information. Nancy McCarroll (Toxicology Branch) presented the mutagenicity data. 6The mode of action (MOA) data on mouse liver tumors was jointly presented by both scientists.

The CARC concluded the following:

Carcinogenicity (Inhalation Study)

Rat (Aiso et al. 2005a)

- No treatment-related tumors were seen in male or female F344 rats.

- Adequacy of Dosing: The CARC considered the highest level tested (300 ppm) to be adequate, but not excessive, to assess the carcinogenicity of *p*-DCB in male and female rats. This conclusion was based on severe effects at 600 ppm seen in a 13-week inhalation toxicity study which included hepatotoxicity (increased liver weight, hepatocellular hypertrophy, increased cholesterol) in male and female rats and renal toxicity (increased kidney weight, ∝2u-globulin nephropathy) in male rats. Similar effects were seen at 270 ppm in the subchronic study but the effects were less severe. The adequacy of dose selection was supported by increased liver and kidney weights as well as non-neoplastic lesions in the liver, kidney, and nasal region seen the 2-year cancer assay at 300 ppm.

Mouse (Aiso et al. 2005a)

- Liver tumors noted in mice at the highest level tested (300 ppm) in both sexes were considered to be treatment-related and a high dose effect since:

  a) In males, there were significant increasing trends in hepatocellular carcinomas and hepatoblastomas, both at $p<0.01$. There was a significant pair-wise comparison of the 300 ppm dose group with the controls for hepatocellular carcinomas [38/49 (78%) vs. 12/49 (24%)], at $p<0.05$. Hepatoblastomas are very rare, malignant tumors.

  b) In females, there were significant increasing trends, and significant pair-wise comparisons of the 300 ppm dose group with the controls, for hepatocellular adenomas [20/50 (40%) vs. 2/50 (4%)] and carcinomas [41/50 (82%) vs. 2/50 (4%)], all at $p < 0.01$. There was also a significant trend in hepatoblastomas at $p < 0.05$.

  c) The incidence of liver tumors in both sexes exceeded published spontaneous rates for liver tumors in the B6C3F1 mice (Haseman et al. 1998; Doull et al., 1983). No historical control data were provided for the BDF1 mice.

- In male mice, there was a significant trend in histiocytic sarcomas in the liver at $p < 0.05$. There was also a significant pair-wise comparison of the 300 ppm dose group with the controls for histiocytic sarcomas in the liver [6/49 (12%) vs. 0/49 (0%)], at $p < 0.05$. When the incidence in the tumors at all sites, not just the liver, was analyzed, there were more tumor bearing mice at the mid dose (16%) than at the high dose (Table 2). No historical control data were provided by the conducting lab but literature values have placed the incidences at 0-6 % (Haseman et al. 1998) to 1-8 % (Giknis and Clifford 2000) for other mouse strains. Even though both the mid and high dose mice exceeded the concurrent control and the historical control range for this tumor type, the CARC did not consider this as treatment related because of the lack of a dose response and the common occurrence and variability of this tumor in many strains of mice.

- Adequacy of Dosing: The CARC considered the highest concentration tested, 300 ppm, to be adequate, but not excessive to assess the carcinogenicity of *p*-DCB via the inhalation route in male and female mice. This conclusion is based on the evidence of severe effects at 600 ppm in a 13-week subchronic inhalation study, which included hepatotoxicity (increased liver weight, hepatocellular hypertrophy) and effects on liver enzymes indicative of liver injury. Similar effects were seen at 270 ppm in the subchronic study but the effects were less severe. This was supported by decreased body weight gain of 12% in males, increased liver and kidney weights seen in both sexes, hepatocellular hypertrophy seen in males, and nasal lesions in females at 300 ppm in the 2-year cancer study.

Previously, the Cancer Peer Review Committee (CPRC) of the HED had met on January 29, 1987 to review the National Toxicology Program (NTP) toxicology database on *p*-DCB with a particular emphasis on the oncogenic potential in rats and mice using the oral route of administration. **These tumor data were not reevaluated at the 2007 meeting, but were carried forward as part of the weight-of- the-evidence evaluation.**

In the NTP chronic rat oral carcinogenicity study, *p*-DCB in corn oil was administered by oral gavage at dosages of 0, 150 or 300 mg/kg/day (males) or 0, 300 or 600 mg/kg/day (females), 5 days/week for 104 weeks to groups of 50 males or 50 females F344/N rats. In the NTP oral carcinogenicity study in the mouse, oral gavage doses of 0, 300 or 600 *p*-DCB in corn oil was administered, 5 days/week for 104 weeks to groups of 50 males or 50 females B6C3F1 mice.

The CPRC concluded the following:

Carcinogenicity (Oral Studies)

Rat

- No significant increases in tumors were observed in females. In males, renal tubular cell adenomas and adenocarcinomas and adenomas/adenocarcinomas combined were significantly elevated in high dose males. The tumorigenic responses exceeded the NTP's average historical control incidences for similar tumors. The kidney tumors induced by p-DCB were not considered relevant to human risk assessment since the induction of the male rat kidney tumors was by the alpha 2u-globulin pathway.

Mouse

- The incidences of hepatocellular adenomas, carcinomas, and adenomas/carcinomas combined were significantly elevated in both males and females of the high dose group. There also were positive trends for all three categories of tumors in both sexes. In addition, four hepatoblastomas, a more uncommon and malignant type of liver tumor than hepatocellular carcinomas, were seen in the high dose males. The CPRC considered these tumors to be treatment-related.

In addition, the CPRC reviewed two inhalation carcinogenicity studies in Wistar rats and SPF Swiss mice, and concluded that inhalation treatment of male and female Wistar rats 5 days /week for 76 weeks at levels as high as 500 ppm did not alter the spontaneous tumor profile in either species under the testing conditions. It was further concluded that the mouse study was inadequate because of study limitations due to an insufficient histopathological evaluation of the tissues.

Mutagenicity

There is no mutagenicity concern for *p*-DCB or the main metabolite (2,5-dichlorophenol).

Structural-Activity Relationship

Although some data were available from the closely related *ortho* and *meta* isomers of *p*-DCB, these data provided very limited support for the carcinogenicity of *p*-DCB.

Mode of Action Analysis

Based on the review of the published literature by HED, the CARC concluded that a plausible non-genotoxic mode of action (MOA) involving mitogenesis was established for the *p*-DCB – induced hepatic tumors in male and female mice. It should also be noted that since tissue distribution of *p*-DCB is considered to be the same, regardless of the route of administration (via inhalation, ingestion, or subcutaneous injection) and the results show consistency in the overall toxicological response, data from both oral and inhalation studies were used to support the MOA analysis. Accordingly, the conclusion for mitogenic stimulation is supported by the following findings from oral and inhalation studies:

1. Neither *p*-DCB nor its main metabolite (2,5-dichlorophenol) is mutagenic. With the exception of single assays showing DNA damage *in vitro* and *in vivo*, data from genetic toxicology studies are negative. These positive results were seen, however, in the absence of a clear mutagenic effect (i.e., gene mutations or chromosome aberrations). Consequently, these data are not sufficient to further consider a mutagenic MOA for the carcinogenic response in mice.

2. There is a good dose correlation between liver tumors, hepatic microsomal enzyme induction and cell proliferation in mice.

3. Cell proliferation occurred in both mice and rats in the absence of overt liver toxicity.

4. Temporal relationships supporting this postulated mitogenic MOA were demonstrated. The mitogenic proliferative response was identified at the tumorigenic dose as early as 1 day in females or 2 days in males after the onset of treatment, and declined after 4 days.

However, as noted above, this response (i.e., increased liver weights, accompanied by stimulation of P-450 microsomal enzymes, cell proliferation, and hepatocellular hypertrophy) was demonstrated in rats, but with less intensity than in mice, and in the absence of tumor formation. Although this information might weaken the argument for the proposed non-genotoxic mechanism for liver tumors in mice, it should be noted that this species difference in the response to *p*-DCB may be attributed to the resistance of rat liver and the susceptibility of the B6C3F1 mouse to liver tumors as previously observed by several authors (Grasso and Hilton, 1991; Eldridge et al., 1992; Haseman et al., 1990). Additionally, *p*-DCB is more extensively metabolized by the B6C3F1 mouse than the F-344 rat.

Classification and Quantification of Carcinogenic Potential

In accordance with the EPA's *Final Guidelines for Carcinogen Risk Assessment* (March 2005), the CARC classified *p*-DCB as **"Not Likely to be Carcinogenic to Humans"** based on convincing evidence that a non-mutagenic MOA involving mitogenesis was established for *p*-DCB induced liver tumors in mice and that the carcinogenic effects are not likely below a defined dose that does not perturb normal liver homeostasis (e.g. increased liver cell proliferation). The quantification of carcinogenic potential is, therefore, not required.

I. INTRODUCTION

On February 21, 2007, the Cancer Assessment Review Committee (CARC) of the Health Effects Division (HED) of the Office of Pesticide Programs (OPP) met to re-evaluate the carcinogenic potential of *para*-Dichlorobenzene (*p*-DCB) (also known as 1,4-dichlorobenzene). This was the third cancer evaluation for this chemical. Para-dichlorobenzene was previously classified in 1989 as a Category C carcinogen. The purpose of this 2007 meeting was to evaluate new inhalation data (an inhalation study in rats and mice by Aiso et al 2005a) and to determine the appropriate cancer classification of this chemical. The status of the rat and mouse tumors that were examined in the previous cancer assessments, and which are not under reexamination in this CARC assessment, was summarized and discussed as part of the weight-of-the-evidence determination of the cancer classification.

para-Dichlorobenzene (*p*-DCB), also known as 1,4-dichlorobenzene (CAS number 106-46-7, PC Code 061501) comes in white crystalline form and is volatile (sublimes easily). It is used as moth repellent, general insecticide, germicide, space odorant. It is also used in the manufacture of 2,5-dichloroaniline, dyes, intermediates, and pharmacy and agricultural products (Lewis, R. J., Sr (Ed). 1993).

II. BACKGROUND INFORMATION

1. Previous Actions

The Cancer Peer Review Committee of the Health Effects Division met on January 29, 1987 to review the toxicology database on *p*-DCB with a particular emphasis on the carcinogenic potential in rats and mice (TXR No. 0052665). The studies that were available for review are discussed below:

A. NTP Oral Carcinogenicity Studies in F344/N Rats and B6C3F1 Mice:

In the rat study, the chemical was administered by gavage in corn oil, at dosage levels of 0, 150, or 300 mg/kg/day in males and 0, 300, or 600 mg/kg/day in females, 5 days/week for 104 weeks. No significant increases in tumors were observed in females. Renal tubular cell adenocarcinomas, and adenocarcinomas and adenomas combined were significantly elevated in males of the high dose group. The increased

tumorigenic responses, seen at the highest dose level, exceeded the NTP's average historical control incidences for similar tumors.

In the mouse study, the chemical was administered by gavage in corn oil, at dosage levels of 0, 300, or 600 mg/kg/day, 5 days/week for 104 weeks. Incidences of hepatocellular adenomas, carcinomas, and adenomas/carcinomas combined were significantly elevated in both males and females of the high dose group. In addition there were positive trends for all three categories of tumors in both sexes. In addition, four hepatoblastomas, a more uncommon and malignant type of liver tumor than hepatocellular carcinomas, were seen in the high dose males.

B. ICI Inhalation Carcinogenicity Studies in Wistar Rats and SPF Swiss Mice:

In the rat study, the chemical was administered to groups of 76 to 79 Alderly Park Wister - derived rats of each sex at concentrations of 0, 75, or 500 ppm in the air, 5 days per week for 76 weeks, and surviving animals were observed for 108 to 112 weeks. The treatment did not alter the spontaneous tumor profile in this species under the testing conditions.

In the mouse study, the chemical was administered to groups of 75 mice of both sexes at concentrations of 0, 75, or 500 ppm in the air for 57 weeks and surviving animals were observed for additional 19 weeks. Limitations regarding insufficient histopathological evaluation of tissues precluded useful interpretation of data from the mouse study.

In this meeting, the Committee members, focusing on the response in the rat study, were equally divided in their opinion as to whether *p*-DCB should be placed in the B2 or C category (Cancer Peer Review Report, Aug 18, 1987, TXR No. 0052665).

Subsequently, on April 20, 1987 the Halogenated Organics Subcommittee of the Science Advisory Board reviewed *p*-DCB and concluded that it should be classified as "Category C" (memo to Hon. Lee Thomas, March 9, 1988 in Cancer Peer Review Report, April 27, 1989, TXR No. 0052666).

The committee's conclusion was based on the following:

a) The absence of a positive response in genotoxicity studies (referring to the kidney tumors in the male rat), *p*-DCB appears to act via an epigenetic mechanism in the male rat rather than through the formation of DNA-adducts.

b) In the case of liver carcinomas in B6C3F1, *p*-DCB and other halogenated compounds may promote the expression of oncogens.

c) The male rat kidney tumors may be the result of a mechanism that would not play a role in humans.

c) There was no support for a higher *p*-DCB classification from human epidemiology studies.

In view of the above data, the HED CPRC agreed that *p*-DCB should be classified as a Category C carcinogen. However, the decision as to whether a low dose extrapolation should be used for risk assessment purposes has been deferred (see Peer Review Report dated April 27, 1989, TXR No. 0052666).

2. Recent Data

Recently, an article was published by S. Aiso et al. (2005a). The authors summarized the findings of carcinogenicity studies in rats and mice with *p*-DCB via inhalation and indicated that the treatment was associated with increased incidences of tumors in mice but not in rats. Detailed considerations of these two studies are given below.

III. EVALUATION OF CARCINOGENICITY STUDIES

1. *Citation: Shigetoshi Aiso, Tetsuya Takeughi, Heihachiro Arito, Kasuke Nagano, Seigo Yamamato, and Taijiro Matsushima. (2005). Carcinogenicity and Chronic Toxicity in Mice and Rats Exposed by Inhalation to para-Dichlorobenzene for Two Years. J. Vet. Med. Sci. 67 (10): 1029, 2005*

A. Experimental Design

Para-Dichlorobenzene (purity greater than 99.9%) was administered by inhalation to groups of 50 F344 rats or 50 BDF1 mice of both sexes at target concentrations of 20, 75, or 300 ppm for 6 hours/day, 5 days/week for two years. A group of 50 rats or 50 mice of either sex served as control. Air concentrations of p-DCB vapor in the exposure chambers were monitored at 15-minute intervals by gas chromatography and were maintained approximately constant (within 1% of the intended concentrations throughout the 2-year exposure).

B. Discussion of Tumor Data

In rats, the treatment did not alter the spontaneous tumor profile in either sex.

In male mice, there were significant increasing trends in hepatocellular carcinomas and hepatoblastomas, both at $p<0.01$. There was also a significant trend in histiocytic sarcomas (liver) at $p<0.05$. There were significant pair-wise comparisons of the 300 ppm dose group with the controls for hepatocellular carcinomas [38/49 (78%) vs. 12/49 (24%)] and histiocytic sarcomas [6/49 (12%) vs. 0/49 (0%)], both at $p<0.05$.

In female mice, there were significant increasing trends, and significant pair-wise comparisons of

the 300 ppm dose group with the controls, for hepatocellular adenomas [20/50 (40%) vs. 2/50 (4%)] and carcinomas [41/50 (82%) vs. 2/50 (4%)], all at $p < 0.01$. There was also a significant trend in hepatoblastomas at $p < 0.05$.

In male mice, there was also a significant trend in histiocytic sarcomas (liver) at $p<0.05$. There was a significant pair-wise comparisons of the 300 ppm dose group with the controls for histiocytic sarcomas [6/49 (12%) vs. 0/49 (0%)], both at $p<0.05$.

Incidences of selected lesions in the livers of mice are shown in Table 1. Incidences of histiocytic sarcoma in different tissues of male and female mice treated with p-DCB are shown in Table 2. Incidences of selected lesions in the livers and kidneys of mice and rats are shown in Table 3.

No historical control data for BDF1 mice were provided by the conducting laboratory for the liver tumors or the histiocytic sarcomas. Historical control incidences for liver tumors and histiocytic sarcomas in B6C3F1 mice from NTP carcinogenicity studies were reported by Haseman et al. 1998. For liver adenomas, carcinomas and hepatoblastomas, the historical control incidences were 4-48%, 9-34%, and 0-2%, respectively for males and 2-40%, 0-38%, and 0 for females. Doull et al. 1983 reported a high and variable spontaneous hepatocellular carcinoma historical control incidence rate for B6F3C1 male mice as 7-55% and for female mice as 2-8%. For histiocytic sarcomas, the historical control incidence was 0-6% for males. Historical control data from Charles River 2000 for histiocytic sarcomas in males was 1-8%.

Table 1: Incidences of hepatocellular lesions in male and female mice treated with p-dichlorobenzene (S. Aiso, personal communication, 2007)

Incidences of hepatocellular neoplasms

p-DCB concentrations	0ppm	20ppm	75ppm	300ppm
Male				
Number of animals examined	49	49	50	49
Number of animals with				
Hepatocellular adenoma(HCA)	13(27%)	9(18%)	7(14%)	13(27%)
Hepatocellular carcinoma(HCC)	12(24%)	17(35%)	16(32%)	38(78%)**↑
Hepatoblastoma(HB)	0(0%)	2(4%)	0(0%)	8(16%)**
Number of animals with				
HCA	8	4	2	4
HCC	7	11	11	23
HB	0	0	0	0
HCA + HCC	5	4	5	7
HCA + HB	0	0	0	0
HCC + HB	0	1	0	6
HCA + HCC + HB	0	1	0	2
Total number of animals with hepatocellular neoplasms	20	21	18	41

** p≤ 0.01 by Fisher's exact test; ↑ p≤ 0.05 by Peto test

p-DCB concentrations	0ppm	20ppm	75ppm	300ppm
Female				
Number of animals examined	50	50	49	50
Hepatocellular adenoma (HCA)	2(4%)	10(20%)	6(12%)	20(40%)**↑↑
Hepatocellular carcinoma (HCC)	2(4%)	4(8%)	2(4%)	41(82%)**↑↑
Hepatoblastoma(HB)	0(0%)	0(0%)	0(0%)	6(12%)*
Number of animals with				
HCA	2	8	5	4
HCC	2	2	1	23
HB	0	0	0	0

HCA + HCC	0	2	1	12
HCA + HB	0	0	0	0
HCC + HB	0	0	0	2
HCA + HCC + HB	0	0	0	4
Total number of animals with hepatocellular neoplasms	4	12	7	45

* $p \leq 0.05$ by Fisher's exact test
** $p \leq 0.01$ by Fisher's exact test; ↑↑ $p \leq 0.01$ by Peto test

Table 2: Incidences of histiocystic sarcoma in different tissues of male and female mice treated with p-Dichlorobenzene (S. Aiso, personal communication, 2007).

Incidences of histiocytic sarcoma in all tissues				
p-DCB concentrations	0ppm	20ppm	75ppm	300ppm
Male				
Number of animals bearing primary histiocytic sarcoma at different sites				
subcutis	0	0	2	0
salivary gland	0	0	1	0
liver	0	3	1	6*↑
urinary bladder	0	0	1	0
epididymis	0	0	1	0
peritoneum	0	0	2	0
Total number of animals bearing primary histiocytic sarcoma	0	3	8	6
Female				
Number of animals bearing primary histiocytic sarcoma at different sites				
subcutis	0	0	0	1
salivary gland	0	1	0	0
liver	2	1	1	0
urinary bladder	1	0	0	0
uterus	11	6	13	12
vagina	1	0	0	0
Total number of animals bearing primary histiocytic sarcoma	15	8	14	13

* $p \leq 0.05$ by Fisher's exact test
↑ $p \leq 0.05$ by Peto test

Table 3: Statistical analysis of incidences of selected lesions in the livers and kidneys of mice and rats exposed by inhalation to p-dichlorobenzene for two years (from S. Aiso et al., 2005a,).

	Male					Female				
p-DCB concentrations	0 ppm	20 ppm	75 ppm	300 ppm	Peto test	0 ppm	20 ppm	75 ppm	300 ppm	Peto test
< Mice >										
Number of animals examined	49	49	50	49		50	50	49	50	
Liver										
Hepatocellular adenoma	13	9	7	13		2	10	6	20 **	↑↑
Hepatocellular carcinoma	12	17	16	38 **	↑	2	4	2	41 **	↑↑
Hepatoblastoma	0	2	0	8 **		0	0	0	6 *	
Histiocytic sarcoma	0	3	1	6 *	↑	2	1	1	0	
Hepatocellular hypertrophy: centrilobular[a)]	0	0	0	34 ##		0	0	0	2	
< Rats >										
Number of animals examined	50	50	50	50		50	50	50	50	
Liver										
Hepatocellular hypertrophy: centrilobular[b)]	0	0	0	5 #		0	0	0	3	
Kidney										
Mineralization: papilla[c)]	0	1	0	41 ##		1	0	0	0	
Urothelial hyperplasia: pelvis[b)]	7	8	13	32 ##		0	0	0	0	
Chronic progressive nephropathy[d)]	50 (3.34)	49 (3.27)	49 (3.35)	50 (3.48)		43 (2.28)	46 (2.15)	45 (2.24)	48 (2.29)	

* and **: Significantly different from control at: $p \leq 0.05$ and $p \leq 0.01$ by Fisher's exact test. ↑ and ↑↑: Significantly different from control at $p \leq 0.05$ and $p \leq 0.01$ by Peto test.

and ##: Significantly different from control at $p \leq 0.05$ and $p \leq 0.01$ by Chi-square test.

a) 25 and 9 males had slight and moderate grades of severity, respectively, in the 300 ppm-exposed male group. One female had slight and the other had moderate grade in the 300 ppm-exposed female group.

b) All cases had slight grade of severity. c) All cases had slight grade of severity except the one having the moderate grade in the 300 ppm-exposed male group.

d) The values in parenthesis indicate the average of severity grade index of the lesion in affected animals. The average of severity grade index are calculated with a following equation. [Σ (grade × number of animals with grade)]÷number of examined animals. Grade: 1=slight, 2=moderate,

C. Non - Neoplastic Lesions, Body and Organ Weights, Food Consumption, and Mortality

In the mouse study, there was no difference in the growth rate between the treated and non-treated mice of either sex except for the 300 ppm male group, highest dose tested, which exhibited significantly ($p \leq 0.01$) decreased body weight gain of 12%, at the end of the 2 years. No difference in food consumption was observed between the treated and the control groups. Absolute and relative liver weights were significantly ($p \leq 0.01$) increased in males and females of the high dose group. Absolute and relative kidney weights in females and relative kidney weights in males were also significantly ($p \leq 0.01$) increased in the high dose group (Table 4).

An increase in the number of males and females bearing liver nodules was observed in both sexes of the high dose group. No significant increase in the incidence of altered cell foci in the liver was observed in either males or females. Increased incidence of centrilobular hypertrophy of hepatocytes was observed in males of the high dose group. There was no histopathological change indicating hepatocellular injury in both sexes of any of the treated groups.

Incidence of the respiratory metaplasia of the nasal gland epithelium increased in females of the high dose (300 ppm), and was significantly increased in the 75 ppm male group. Increased

incidence of the respiratory metaplasia of the olfactory epithelium with slight severity was noted in males of the 75 ppm group. The respiratory metaplasia was manifested as replacement of the olfactory epithelium with the respiratory-like mucosal epithelium in which those epithelial cells were similar to the ciliated cells of normal respiratory epithelium. Table 5 shows the nasal lesions in animals exposed to p-DCB for two years.

Survival rates decreased in all treated male groups (39/49, 31/49, 32/50, and 30/49 in the control, 20 ppm, 75 ppm, and 300 ppm, respectively). The decrease in survival rate did not appear to be dose-dependent. However, according to the author, only the decrease in the survival rate of the 300-ppm male group was significant in the Kaplan-Meier survival analysis. A total of 19 deaths consisting of 12 liver tumors deaths, 3 other tumor deaths and 4 non-neoplastic deaths occurred in the 300 ppm group while 3 liver tumor deaths, 3 other tumor deaths and 4 non-neoplastic deaths were observed in the control group. There was no difference in survival rate between the treated female groups and the control group (28/50, 25/50, 23/49, 26/50 for the control, 20, 75, and 300 ppm respectively).

In the rat study, significant ($p \leq 0.01$) increases in absolute and relative organ weights were observed in the liver of males and females and in the kidney of males of the high dose group. No macroscopic lesions were observed in the organs of any of the treated groups. There were no significant increases in the incidences of the neoplastic or in the tumor-related lesions observed in any organ of the treated groups (Table 4).

Incidence of centrilobular hypertrophy of hepatocytes was increased in the males of the high dose group. Increased incidences of papillary mineralization and hyperplasia of the pelvic urothelium in the kidney were observed in males of the high group. No histopathological changes indicative of $\alpha_{2\mu}$ - globulin in the epithelial cells of renal proximal tubules were observed in the inhalation carcinogenicity study, although both the hyaline droplets and the granular casts in the proximal tubules of male rats were noted earlier in the thirteen week inhalation study.

Incidences of the eosinophilic globules in the nasal cavity were increased in the olfactory epithelium with marked grade of severity in the females of the middle and high dose groups and in the respiratory epithelium having slight grade of severity in the high dose females. The increased incidences of the eosinophilic globules were closely associated with a marked decrease in the number of olfactory cells in the olfactory epithelium of 300 ppm-exposed females. Incidence of the respiratory metaplasia of the nasal gland epithelium was increased in the females exposed to 300 ppm. The eosinophilic globules were abundantly present in both the supporting cells of the olfactory epithelium and the ciliated and non-ciliated cells of the respiratory epithelium, (Table 5).

No significant decrease in survival rate was observed in the treated groups of either sex when compared to the control rats, except for the males of the 300 ppm group as indicated by Logrank

analysis (33/50, 34/50, 29/50, 18/50 for the control, 20 ppm, 75 ppm, and 300 ppm respectively). The cause of death in the males of the 300 ppm group was as follows: 10 animals due to leukemia, 9 animals due to other tumors of various organs, 11 animals due to chronic progressive nephropathy (CPN) and 2 animals with unconfirmed cause. The cause of death of the 17 animals in the control group was as follows: 3 animals due to leukemia, 8 animals due to other tumors and 6 CPN. Therefore the significant decrease in the survival rate of males of the 300 ppm group was attributed to an increase in the number of leukemia and CPN deaths.

Table 4: Body and organ weight changes of mice and rats exposed by inhalation to p-DCB for 2 years (from S. Aiso et al. 2005a).

		Male				Female			
p-DCB concentrations		0 ppm	20 ppm	75 ppm	300 ppm	0 ppm	20 ppm	75 ppm	300 ppm
<Mice>									
Number of animals examined[a]		39	31	32	30	28	25	23	26
Body weight	g[b]	42.5±8.1	40.3±8.4	41.6±6	37.6±3.9**	32.4±5.4	34.3±6.1	31.2±5.3	29.6±2.3
Liver	g[b]	1.700	1.908	1.984	3.159 **	1.583	1.857	1.571	5.354**
		± 0.45	± 0.616	± 0.756	± 0.163	± 0.429	± 1.283	± 0.433	± 2.955
	%[c]	4.212	5.062	4.868	8.606**	5.010	5.620	5.020	17.947**
		± 1.776	± 2.395	± 2.1	± 4.766	± 1.703	± 4.412	± 0.791	± 8.83
Kidneys	g[b]	0.674	0.637	0.655	0.722	0.467	0.469	0.457	0.514 *
		± 0.266	± 0.071	± 0.064	± 0.346	± 0.093	± 0.045	± 0.062	± 0.112
	%[c]	1.686	1.655	1.605	1.955 **	1.474	1.396	1.480	1.749**
		± 1.082	± 0.438	± 0.283	± 1.1	± 0.378	± 0.22	± 0.156	± 0.433
<Rats>									
Number of animals examined[a]		33	34	29	18	38	34	38	36
Body weight	g[b]	394	406	382	382	300	304	298	295
		± 40	± 45	± 42	± 43	± 31	± 40	± 45	± 42
Liver	g[b]	13.007	14.309	13.773	14.957**	7.864	8.032	8.193	9.055**
		± 1.669	± 4.983	± 2.357	± 1.883	± 0.899	± 1.187	± 1.378	± 1.681
	%[c]	3.331	3.583	3.634	3.950**	2.644	2.657	2.774	3.159**
		± 0.546	± 1.375	± 0.69	± 0.562	± 0.379	± 0.33	± 0.394	± 0.901
Kidneys	g[b]	3.040	3.183	3.193	3.636**	2.088	2.042	2.081	2.172
		± 0.261	± 0.59	± 0.352	± 0.493	± 0.178	± 0.175	± 0.182	± 0.23
	%[c]	0.779	0.800	0.849	0.967**	0.703	0.680	0.715	0.758
		± 0.108	± 0.204	± 0.165	± 0.194	± 0.09	± 0.093	± 0.134	± 0.186

a) The number of animals at terminal necropsy. b) Absolute organ weights are expressed as means ± SD.
c) Relative organ weights, the percentage of absolute organ weight to body weight are expressed as means ± SD.
* and **: significantly different from control at $p \leq 0.05$ and $p \leq 0.01$ by Dunnett's test, respectively.

Table 5: Nasal lesions in mice and rats exposed by inhalation to p-DCB for 2 years (from S. Aiso et al. 2005a)

p-DCB concentrations		Male				Female			
		0 ppm	20 ppm	75 ppm	300 ppm	0 ppm	20 ppm	75 ppm	300 ppm
< Mice>									
No. of animals examined		49	49	50	49	50	50	49	50
Respiratory metaplasia									
Nasal gland	T[a]	37	42	47 *	41	9	6	8	19
	(1+)	(28)	(29)	(27)	(30)	(9)	(6)	(8)	(18)
	(2+)	(9)	(12)	(18)	(11)	(0)	(0)	(0)	(1)
	(3+)	(0)	(1)	(2)	(0)	(0)	(0)	(0)	(0)
Olfactory epithelium	T[a]	23	30	38 **	24	7	6	2	20 **
	(1+)	(23)	(30)	(37)	(22)	(7)	(6)	(2)	(20)
	(2+)	(0)	(0)	(1)	(2)	(0)	(0)	(0)	(0)
< Rats>									
No. of animals examined		50	50	50	50	50	50	50	50
Eosinophilic globules									
Respiratory epithelium	T[a]	4	1	5	4	11	10	14	38 **
	(1+)	(4)	(1)	(5)	(4)	(11)	(10)	(14)	(38)
Olfactory epithelium	T[a]	33	22	21	26	49	46	46 **	50 **
	(1+)	(32)	(20)	(19)	(19)	(22)	(17)	(7)	(3)
	(2+)	(1)	(1)	(1)	(7)	(21)	(27)	(16)	(27)
	(3+)		(1)	(1)	(0)	(6)	(2)	(23)	(20)
Respiratory metaplasia									
Nasal gland	T[a]	3	0	0	0	5	4	4	33 **
	(1+)	(3)	(0)	(0)	(0)	(5)	(4)	(4)	(33)

a) Total number of animals bearing the nasal lesion.
* and **: Significantly different from control at p≤0.05 and p≤0.01 by Chi Square test.

D. Adequacy of the Dosing for Assessment of Carcinogenicity

The selection of the dose levels used in these two inhalation studies in rats and mice was based on the results of a 13-week inhalation toxicity study of p-dichlorobenzene in mice and rats (S. Aiso et al., 2005b). In this short-term study, the animals were exposed by inhalation to *p*-DCB at concentration levels of 0, 25, 55, 120, 270 or 600 ppm, 6 hours/day, 5 days/week for 13 weeks. Control groups were also included and exposed to clean air under the same conditions.

The treatment decreased the growth rate of male mice, and induced hepatotoxicity in mice and rats of both sexes. The induced hepatotoxicity at 600 ppm was severe and manifested as increased liver weight, hepatocellular hypertrophy, and increased serum levels of total cholesterol. Liver necrosis and increased serum levels of AST and ALT, indicative of hepatocellular death were observed in the mice, but not in rats. Similar effects were seen at 270 ppm but were not severe.

Treatment at 600 ppm also caused renal and hematological toxicity in male but not female rats or mice of either sex. Renal lesions were evidenced by the hyaline droplets, positive for $\alpha_{2\mu}$ -

globulin, observed in the proximal tubular epithelial cells. Granular casts were observed in the tubular lumen, resulting from the necrotic desquamation of the renal tubular epithelium. Papillary mineralization in the renal pelvis and increased serum levels of BUN and creatinine were observed. These renal changes are indicative of $\alpha_{2\mu}$ - globulin nephropathy. Hematological toxicity in male rats was manifested as decreases in red blood cell counts, hemoglobin concentration, hematocrit and mean corpuscular volume and increased spleen weight. Similar effects were seen at 270 ppm but were not severe.

The NOAEL was considered by the authors to be 120 ppm. The LOAEL was 270 ppm for the hepatotoxicity in mice and for renal toxicity in rats. Based on this study, the maximum tolerated dose for the 2-year inhalation carcinogenicity studies in both rats and mice was estimated to be 300 ppm.

From the above assessment, the CARC concluded that the highest concentration tested (300 ppm) in both rats and mice was adequate, but not excessive, to assess the carcinogenicity of *p*-DCB in both species. In rats, this conclusion was based on severe effects at 600 ppm seen in a 13-week inhalation toxicity study which included hepatotoxicity (increased liver weight, hepatocellular hypertrophy, increased cholesterol) in male and female rats and renal toxicity (increased kidney weight, ∝2u-globulin nephropathy) in male rats. Similar effects were seen at 270 ppm in the subchronic study, but the effects were less severe. The adequacy of dose selection was supported by increased liver and kidney weights as well as non-neoplastic lesions in the liver, kidney, and nasal region seen the 2-year rat cancer assay at 300 ppm. In mice, justification for selection of 300 ppm as the high concentration is based on the evidence of severe effects at 600 ppm in a 13-week subchronic inhalation study. This included hepatotoxicity (increased liver weight, hepatocellular hypertrophy) and effects on liver enzymes indicative of liver injury. Similar effects were seen at 270 ppm in the subchronic study but the effects were less severe. This was supported by decreased body weight gain of 12% in males, increased liver and kidney weights seen in both sexes, hepatocellular hypertrophy seen in males, and nasal lesions in females at 300 ppm in the 2-year cancer study.

IV. TOXICOLOGY

1. Metabolism

The pharmacokinetics of *p*-DCB was evaluated in female adult CFY-Sprague-Dawley rats. Groups of two animals per dose received ^{14}C-labeled *p*-DCB at doses of 250 mg/kg/day either orally by gavage, subcutaneously for up to 10 days, or via inhalation at a concentration level of 1000 ppm 3 hours/day, for up to 10 days. Based on the findings of this study, it was concluded that similar tissue distribution, metabolism, and excretion patterns occurred for p-DCB by all three routes of administration. The highest levels of radioactivity were found in fat followed by kidney, lung and liver, plasma, and muscle. The peak tissue concentrations occurred in about 6 days. By 8 hours after the last of the repeated doses, both tissue and plasma levels declined and

were largely undetectable after 5 days. Over 90 percent of the radioactivity was excreted in the urine by all three routes as sulfate and glucuronide conjugates of 2,5-dichlorophenol (Cancer Peer Review Report dated August 18, 1987, citing Xenobiotica 10:81-95, 1980).

The conversion of *p*-DCB to 2,5-dichlorophenol and subsequent conjugation was confirmed in several other studies. According to a document entitled, *Public Health Goal for 1,4-Dichlorobenzene in Drinking Water,* prepared by CA EPA, 1997, p-DCB is converted into exposed humans to 2,5-dichlorophenol, which then undergoes conjugation before it is excreted as glucuronide and sulphate conjugates, and 2,5-dichloroquinone (NTP, 1987; citing Hallowell, 1959; Pagnotto and Walkley, 1965). The 2,5-dichlorophenol has been detected in the urine of exposed humans (HSBD, 1997; citing Menzie, 1969).

The biotransformation and tissue distribution of *p*-DCB was compared in rats and rabbits. The primary metabolite of *p*-DCB detected in the urine of rats exposed orally to p-DCB was 2,5-dichlorophenyl methyl sulfone. Rabbits exposed orally to p-DCB formed 2,5-dichlorophenol (plus glucuronide and ethereal sulphate conjugates) and 2,5-dichloroquinol (HSBD 1997; citing Azouz et al., 1955). Similarly, rats exposed to ^{14}C-labeled *p*-DCB excreted most of the dose as sulphate or glucuronide conjugates of 2,5-dichlorophenol (Klos and Dekant, 1994; NTP citing Hawkins et al., 1980). Minor metabolites reported in rats include 2-(N-acetyl-cysteine-S-yl)-1, 4-dichlorobenzene and 2-(N-acetyl-cysteine-S-yl)-2,3-dihydro-3-hydroxy-1,4-dichlorobenzene (Klos and Dekant, 1994)."

When several species and strains of animals (including humans) were compared in the ability of their hepatic microsomes to biotransform *p*-DCB (Hissink et al., 1997), it was found that B6C3F1 mouse microsomes showed the greatest ability to metabolize p-DCB, followed by the rat then human microsomes. The authors measured the extent of conversion of radiolabeled *p*-DCB to oxidized metabolites and glutathione conjugates and the extent of covalent binding of radioactivity (i.e. metabolites) to microsomal proteins. As summarized in Table 6, *p*-DCB was extensively metabolized by the B6C3F1 mouse (15% of total radioactivity) compared to the Fischer-344 rat (1.1%) or the Wistar rat (1.3%). Furthermore, the extent of covalent binding of radioactivity (as % of total radioactivity) by the mouse microsomes was 31 to 39 times greater than the rat (Table 6), when % of conversion to metabolites and the % of metabolites bound are taken together. Using ascorbic acid, an inhibitor of hydroquinone oxidation to benzoquinones, the authors attributed the more extensive binding in the mouse to the greater formation of benzoquinones by the mouse microsomes than by those in the rat. These authors concluded that the more pronounced conversion of *p*-DCB to reactive species, including benzoquinones, could be a factor in its hepatocarcinogenicity in mice but not in rats.

Table 6. In vitro metabolism of 1,4-DCB by rat and mouse liver microsomes[1]

Species	Conversion (% of Total Radioactivity)	Covalent Binding		Identified Metabolites (% of Total Conversion)			
		As % of total Conversion	As % of total Radioactivity [2]	GS-Epoxide	Hydroquinone	2,5-DCP	GS-Quinone
B6C3F1-Mouse	15	20.9	3.1	ND	16.1	50.5	3.0
Fischer 344-Rat	1.1	6.8	0.08	5.0	27.1	56.8	6.0
Wistar-Rat	1.3	8.1	0.1	15.0	10.5	50	ND

[1] Data from Hissink et al. (1997)
[2] Binding (as % of total radioactivity) = Binding (as % of total conversion) x Conversion (as % of total radioactivity), calculations were not in the original report, were added by the reviewer.

According to a document prepared by the National Institute for Working Life of Sweden (Lundberg, 1998) there are no quantitative data available on the tissue distribution of p-DCB in human subjects. However, small amounts of *p*-DCB have been found in blood, fatty tissue and breast milk of subjects exposed environmentally to p-DCB. In rats given radiolabeled *p*-DCB, the highest concentrations of radioactivity were recovered in fat, kidneys and liver, while the lowest amounts were recovered in the lungs, muscles and plasma. The distribution was the same regardless of the method of administration whether via inhalation, ingestion or subcutaneous injection. It is of interest also that rats showed some gender differences in distribution of *p*-DCB in kidneys and liver. These gender differences are probably related to the nephrotoxic effects observed in males and hepatotoxic effects seen in females.

Dichlorobenzenes are generally metabolized in three phases; oxidation catalyzed by cytochrome p-450, followed by conjugation reactions, and enterohepatic circulation of metabolites and their conversion by intestinal enzymes. Figure 1 shows the biotransformation of *p*-DCB. Figure 1 below shows a proposed schematic illustration of oxidation of *p*-DCB by the microsomal enzymes.

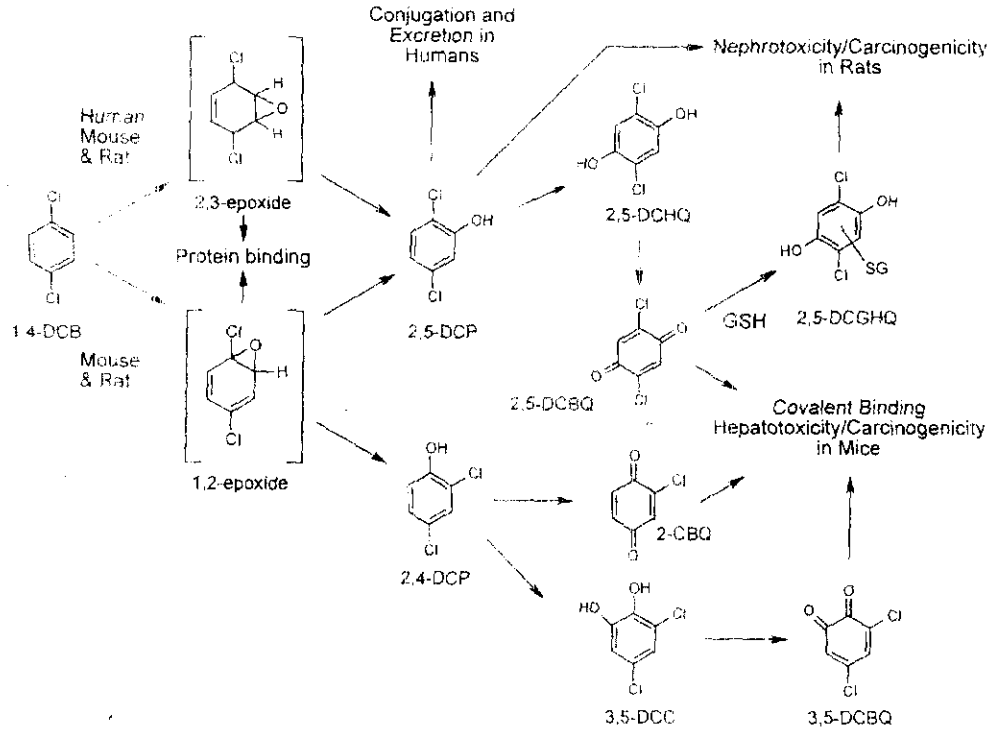

Figure 1: Biotransformation of p-Dichlorobenzene. Pathways for the formation of reactive metabolites by mouse, rat and human microsomes and their proposed effects are shown. [From Muller (2002)]

2. Mutagenicity

p-DCB has been classified as not mutagenic in the standard battery of Genetic Toxicology studies by IARC (1987) and the Cancer Peer Review Committee of HED (1987). Since that time, several articles have appeared in the open literature. These studies indicate that neither the parent compound nor its main metabolite (2,5-dichlorophenol) were mutagenic in the Chinese hamster ovary (CHO) cell gene mutation assay or induced micronuclei in the bone marrow of NMRI male or female mice using oral gavage administration of 2500 mg/kg 1,4-DCB or 1500 2,5-DCP (Tegethoff et al., 2000). It is of note that in this assay, the negative induction of micronuclei was accompanied by decreases in polychromatic erythrocytes (PCE). The same authors also used the i.p. route to expose mice to 2 x 177.5 and 2 x 355 mg/kg and found that 1,4-DCB failed to induce a positive effect. Similarly, Sherman et al. (1998) demonstrated that p-DCB did not induce unscheduled DNA synthesis in F-344 rats kidney cells or B6C3F1 mouse hepatocytes following *in vivo* exposure. These authors, however, noted increased scheduled DNA synthesis, indicative of cell proliferation at single oral gavage doses up to a level (1000 mg/kg) higher than the tumorigenic dose in the 1987 NTP study (600 mg/kg/day). There is high confidence in the findings of Tegethoff et al. and Sherman et al. since clear interaction with the

target organ occurred in the absence of a positive response. *p*-DCB was also found to be negative in both rat and human primary hepatocytes for DNA damage in the alkaline elution assay (Canonero et al., 1997). In contrast to these negative findings, these authors reported positive results in an *in vitro* micronucleus assay in primary rat hepatocytes but the response was not dose related; only observed at single concentrations in different trials; and not seen in primary cultures of human hepatocytes or in mouse bone marrow *in vivo*. Sasaki et al. (1997) also reported that p-DCB was reactive in the alkaline single-cell gel electrophoresis (Comet) assay in mouse liver 3 hours following an i.p. injection with 2000 mg/kg. At 24 hours, however, the response was negative. Carbonell et al. (1991) reported the induction of sister chromatid exchange (SCE) in human lymphocytes from two separate donors at 0.1 and 0.2 µg/mL. While both the SCE assay (*in vitro*) and the comet assay (*in vivo*) measure reactivity with DNA, these positive results are not convincing. In the absence of a clear mutagenic response (i.e., positive gene mutations or chromosome aberrations) the findings are not sufficient to further consider a mutagenic MOA for *p*-DCB.

3. Structure-Activity Relationship

The structure-activity relationship was addressed by the CPRC of the HED in the previous meeting. According to the CPRC's report of August 18, 1987, data from close structure analogues provided very limited support for the carcinogenicity of p-DCB. Since that time, additional information has come to light. Both the *ortho* and *meta* isomers of dichlorobenzene are well-known industrial chemicals that are toxic to the liver (*o*-DCB is the most toxic followed by *m*-DCB, and *p*-DCB is the least toxic) (Stine et al., 1991 as cited by Umemura et al., 1996). IRIS (2006) lists *o*-DCB as Group 3, based on the lack of increased tumor induction in well-conducted mouse and rat 2-year carcinogenicity studies. *m*-DCB has not been adequately tested for potential carcinogenicity. According to IRIS, both the *ortho* and the *meta* isomers induced micronuclei in the bone marrow of mice treated *in vivo*; neither compound is mutagenic to bacteria and both produced mixed results in DNA binding assays. Umemura et al. (1996) showed that both the o- and the m-forms produce isomer specific acute toxicity to the mouse liver as indicated by significant increases in liver weight, ALT activity and BrdU labeling as well as extensive liver necrosis. In contrast, the BrdU labeling seen in this study for *p*-DCB (discussed in the MOA attachment) occurred only at doses showing little or no liver necrosis. Based on the findings, the authors ranked the three isomers according to hepatotoxicity as: >*m*-DCB >*o*-DCB >*p*-DCB.

4. Subchronic Toxicity

Thirteen-week inhalation toxicity study of p-dichlorobenzene in mice and rats (S. Aiso et al. J. Occup Health. 2005b)

This study has been already discussed under "Section III-D, Adequacy of the Dosing for Assessment of Carcinogenicity"

5. Mode of Action Analysis for Liver Tumors in Mice

Since *p*-DCB is predominantly negative in a wide range of genotoxicity studies and is not overtly cytotoxic to the liver, the possibility that this compound is a rodent carcinogen via a mitogenic mode of action (MOA) was explored by HED toxicologists. Based on the available information in the published literature, it is postulated that *p*-DCB causes liver tumors in male and female mice through chronic exposure to inhalation and/or oral doses that cause stimulation of mitogenesis leading to cell proliferation and progressing to adenomas and carcinomas (see Attachment).

Based on the review of the published literature by HED, the CARC concluded that a plausible non-genotoxic mode of action (MOA) involving mitogenesis was established for the *p*-DCB – induced hepatic tumors in male and female mice. It should also be noted that since tissue distribution of p-DCB is considered to be the same, regardless of the route of administration (via inhalation, ingestion, or subcutaneous injection) and the data show consistency in the overall toxicological response, data from both oral and inhalation studies were used to support the MOA analysis. Accordingly, the conclusion for mitogenic stimulation is supported by the following findings from oral and inhalation studies:

1. Neither *p*-DCB nor its main metabolite (2,5-dichlorophenol) are genotoxic. With the exception of single assays showing DNA reactivity *in vitro* and *in vivo*, data from genetic toxicology studies are negative. These positive results were seen, however, in the absence of a clear mutagenic effect (i.e., gene mutations or chromosome aberrations). Consequently, these data are not sufficient to further consider a mutagenic MOA for the carcinogenic response in mice.

2. There is a good dose correlation between liver tumors, hepatic microsomal enzyme induction and cell proliferation in mice.

3. Cell proliferation occurred in both mice and rats in the absence of overt liver toxicity.

4. Temporal relationships supporting this postulated mitogenic MOA were demonstrated. The mitogenic proliferative response was identified at the tumorigenic dose as early as 1 day in females or 2 days in males after the onset of treatment, and declined after 4 days.

As noted above, this response (i.e., increased liver weights, accompanied by stimulation of P-450 microsomal enzymes, cell proliferation, and hepatocellular hypertrophy) was also demonstrated in rats, but with less intensity than in mice, and in the absence of tumor formation. Although this information might weaken the argument for the proposed non-genotoxic mechanism for liver tumors in mice, it should be noted that this species difference in the response to *p*-DCB may be attributed to the resistance of rat liver and the susceptibility of the B6C3F1 mouse to liver tumors as previously observed by several

authors (Grasso and Hilton, 1991; Eldridge et al., 1992; Haseman et al., 1990).

V. COMMITTEE'S ASSESSMENT OF THE WEIGHT-OF-THE-EVIDENCE

The Committee's assessment of the weight-of-the-evidence is discussed below:

1. Carcinogenicity

Inhalation Study

Rat (Aiso et al. 2005a)

- No treatment-related tumors were seen in male or female F344 rats.

- Adequacy of Dosing: The CARC considered the highest level tested (300 ppm) to be adequate, but not excessive, to assess the carcinogenicity of *p*-DCB in male and female rats. This conclusion was based on severe effects at 600 ppm (significant effects on liver and kidney weights) in a 13-week inhalation study in conjunction with non-neoplastic lesions in the nasal region, which was supported, by similar lesions in the 2-year cancer assay at 300 ppm.

Mouse (Aiso et al. 2005a)

- Liver tumors noted at the highest level tested (300 ppm) in both sexes were considered to be treatment-related and a high dose effect since:

  a) In male mice, there were significant increasing trends in hepatocellular carcinomas and hepatoblastomas, both at $p<0.01$. There was a significant pair-wise comparison of the 300 ppm dose group with the controls for hepatocellular carcinomas [38/49 (78%) vs. 12/49 (24%)], at $p<0.05$. Hepatoblastomas are very rare, malignant tumors.

  b) In female mice, there were significant increasing trends, and significant pair-wise comparisons of the 300 ppm dose group with the controls, for hepatocellular adenomas [20/50 (40%) vs. 2/50 (4%)] and carcinomas [41/50 (82%) vs. 2/50 (4%)], all at $p < 0.01$. There was also a significant trend in hepatoblastomas at $p < 0.05$.

  c) The incidence of liver tumors in both sexes exceeded published spontaneous rates for liver tumors in the B6C3F1 mice (Haseman et al. 1998; Doull et al., 1983). No historical control data from the conducting lab were provided for the BDF1 mice.

- In male mice, there was a significant trend in histiocytic sarcomas in the liver at $p < 0.05$. There was also a significant pair-wise comparison of the 300 ppm dose group with the controls for histiocytic sarcomas in the liver [6/49 (12%) vs. 0/49 (0%)], at $p < 0.05$. When the incidence in the tumors at all sites, not just the liver, was analyzed, there were more tumor bearing mice at the mid dose (16%) than at the high dose (Table 2). No historical control data were provided by the conducting lab but literature values have placed the incidences at 0-6 % (Haseman et al. 1998) to 1-8 % (Giknis and Clifford 2000) for other mouse strains. Even though both the mid and high dose mice exceeded the concurrent control and the historical control range for this tumor type, the CARC did not consider this as treatment related because of the lack of a dose response and the common occurrence and variability of this tumor in many strains of mice.

Previously, the Cancer Peer Review Committee (CPRC) of the HED had met on January 29, 1987 to review the National Toxicology Program (NTP) toxicology database on *p*-DCB with a particular emphasis on the oncogenic potential in rats and mice using the oral route of administration. **These tumor data were not reevaluated at the 2007 meeting, but were carried forward as part of the weight-of- the-evidence.**

In the NTP chronic rat oral carcinogenicity study, *p*-DCB in corn oil was administered by oral gavage at dosages of 0, 150 or 300 mg/kg/day (males) or 0, 300 or 600 mg/kg/day (females), 5 days/week for 104 weeks to groups of 50 males or 50 females F344/N rats. In the NTP oral carcinogenicity study in the mouse, oral gavage doses of 0, 300 or 600 p-DCB in corn oil was administered, 5 days/week for 104 weeks to groups of 50 males or 50 females B6C3F1 mice.

The CPRC concluded the following:

Carcinogenicity (Oral Studies)

Rat

- No significant increases in tumors were observed in females. In males, renal tubular cell adenomas and adenocarcinomas and adenomas/adenocarcinomas combined were significantly elevated in high dose males. The tumorigenic responses exceeded the NTP's average historical control incidences for similar tumors. The kidney tumors induced by p-DCB were not considered relevant to human risk assessment since the induction of the male rat kidney tumors was by the alpha 2u-globulin pathway.

Mouse

- The incidences of hepatocellular adenomas, carcinomas, and adenomas/carcinomas combined were significantly elevated in both males and females of the high dose group. There also were positive trends for all three categories of tumors in both sexes. In

addition, four hepatoblastomas, a more uncommon and malignant type of liver tumor than hepatocellular carcinomas, were seen in the high dose males.

In addition, the CPRC reviewed two inhalation carcinogenicity studies in Wistar rats and SPF Swiss mice, and concluded that inhalation treatment of male and female Wistar rats 5 days /week for 76 weeks at levels as high as 500 ppm did not alter the spontaneous tumor profile in either species under the testing conditions. It was further concluded that the mouse study was inadequate because of study limitations due to an insufficient histopathological evaluation of the tissues.

2. Mutagenicity

There is no mutagenicity concern for *p*-DCB or the main metabolite (2,5-dichlorophenol).

3. Structural-Activity Relationship

Although some data were available from the closely related *ortho* and *meta* isomers of *p*-DCB, these data provided very limited support for the carcinogenicity of *p*-DCB.

4. Mode of Action Analysis

Based on the review of the published literature by HED, the CARC concluded that a plausible non-genotoxic mode of action (MOA) involving mitogenesis was established for the *p*-DCB – induced hepatic tumors in male and female mice. It should also be noted that since tissue distribution of p-DCB is considered to be the same, regardless of the route of administration (via inhalation, ingestion, or subcutaneous injection) and the data show consistency in the overall toxicological response, data from both oral and inhalation studies were used to support the MOA analysis. Accordingly, the conclusion for mitogenic stimulation is supported by the following findings from oral and inhalation studies:

- Neither *p*-DCB nor its main metabolite are genotoxic. With the exception of single assays showing DNA reactivity *in vitro* and *in vivo*, data from genetic toxicology studies are negative. These positive results were seen, however, in the absence of a clear mutagenic effect (i.e., gene mutations or chromosome aberrations). Consequently, these data are not sufficient to further consider a mutagenic MOA for the carcinogenic response in mice.

- There is a good dose correlation between liver tumors, hepatic microsomal enzyme induction and cell proliferation in mice.

- Cell proliferation occurred in both mice and rats in the absence of overt liver toxicity.

- Temporal relationships supporting this postulated mitogenic MOA were demonstrated. The mitogenic proliferative response was identified at the tumorigenic dose as early as 1 day in females or 2 days in males after the onset of treatment, and declined after 4 days.

As noted above, this response (i.e., increased liver weights, accompanied by stimulation of P-450 microsomal enzymes, cell proliferation, and hepatocellular hypertrophy) was also demonstrated in rats, but with less intensity than in mice, and in the absence of tumor formation. Although this information might weaken the argument for the proposed non-genotoxic mechanism for liver tumors in mice, it should be noted that this species difference in the response to *p*-DCB may be attributed to the resistance of rat liver and the susceptibility of the B6C3F1 mouse to liver tumors as previously observed by several authors (Grasso and Hilton, 1991; Eldridge et al., 1992; Haseman et al., 1990). In addition, the findings from the metabolism study of Hissink etal. (1997), showing that p-DCB is extensively metabolized by the B6C3F1 mouse (15% of total radioactivity) as compared to the F-344 rat (1.1%), may be a factor influencing hepatocarcinogenicity in the mouse but not the rat.

VI. CLASSIFICATION OF CARCINOGENIC POTENTIAL

In accordance with the EPA's *Final Guidelines for Carcinogen Risk Assessment* (March 2005), the CARC classified p-DCB as **"Not Likely to be Carcinogenic to Humans"** based on convincing evidence that a non-mutagenic MOA involving mitogenesis was established for *p*-DCB induced liver tumors in mice and that the carcinogenic effects are not likely below a defined dose that does not perturb normal liver homeostasis (e.g., increased liver cell proliferation).

VII. QUANTIFICATION OF CARCINOGENIC POTENTIAL

The quantification of carcinogenic potential is not required.

VIII. BIBLIOGRAPHY

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ATTACHMENT

DETAILED MODE of ACTION ANALYSIS

MODE of ACTION ANALYSIS for LIVER TUMORS in MICE

Description of the postulated mode of action

Since p-DCB is predominantly negative in a wide range of genotoxicity studies and is not overtly cytotoxic to the liver, the possibility that this compound is a rodent carcinogen via a mitogenic mode of action (MOA) was explored by HED toxicologists. Based on the available information in the published literature, it is postulated that p-DCB causes liver tumors in male and female mice through chronic exposure to inhalation and/or oral doses that cause stimulation of mitogenesis leading to cell proliferation and progressing to adenomas and carcinomas.

Key events

The postulated key precursor events that may be associated with liver tumor formation by p-DCB involve: induction of P-450 microsomal enzymes leading to mitogenic stimulation, which influences the number of cell divisions and promotes cell proliferation, as indicated by increased BrdU labeling. The sequence of events continues to hepatocellular hypertrophy, increased organ weights and ultimately culminates in liver adenomas and carcinomas (Figure 1).

Figure 1. Proposed Key Parameters involved in Mitogenic Stimulation in the Mode of Action Analysis for p-DCB

Key Parameters Supportive of Mitogenic Stimulation:
1. Induction of P-450 microsomal enzymes
2. Cell proliferation (increased BrdU labeling), manifested by hepatocellular hypertrophy and increased liver weights
3. Liver tumors

Studies Supporting Mitogenic Stimulation as the MOA: Induction of P-450 Microsomal Enzymes, Cell Proliferation, Increased Liver Weights, and Hypertrophy

MOA studies were found in the open literature on the oral exposure route; in addition to the 2-year inhalation study, a 13-week inhalation study was also available from the open literature. It was found that the tissue distribution of *p*-DCB is the same regardless of the route of administration (via inhalation, ingestion or subcutaneous injection) and the data show consistency in the toxicological response between oral and inhalation exposures. Consequently, the MOA for both exposure routes is considered; oral studies are presented first followed by the inhalation studies.

Oral Studies

In the study of Lake et al. (1997), B6C3F1 male mice received daily oral gavage doses of 0, 300 or 600 mg/kg and F344 male rats were dosed with 0, 25, 75, 150 or 300 mg/kg/day *p*-DCB daily in corn oil (5 days/week) for 1, 4, or 13 weeks. In general agreement with the other findings that will be discussed later, significant and dose-related increases in relative liver weights of male rats and mice Figure 2 (Figure 1 from the report) which were associated with mild centrilobular hypertrophy (300 mg/kg/day, rats at 13 weeks) or marked centrilobular hypertrophy (600 mg/kg/day, mice at 13 weeks) were reported. Replicative DNA synthesis data were also consistent with other reports. As shown in Figure 3 (Figure 2 from the study report) a significant increase (255% ↑) in BrdU labeling of hepatocytes was observed from rats treated with 300 mg/kg/day for 1 week; no other significant effects were seen at any dose or sacrifice time. By contrast, mice showed significant increases (475 and 1175% ↑) in BrdU labeling of hepatocytes at 300 and 600 mg/kg/day, respectively at week 1. The response was sustained but less intense at week 4 with significant increases (420 and 395% ↑) occurring at 300 and 600 mg/kg/day; and a significant increase (≈ 200% ↑) persisted through week 13 at 600 mg/kg/day.

In addition to these parameters, the investigators also evaluated cytochrome P-450 content and the mixed function oxidase (MFO) activity of 7-pentoxyresorufin O-dependentylase (PROD, marker for CYP2B subfamily of isoenzymes) and found a sustained induction of P-450 content and PROD Figure 4 (of the study report). In rats, the P-450 response was significant and dose-related at 150 and 300 mg/kg/day (week 1), at 25- 300 mg/kg/day (week 4) and at 75-300 mg/kg/day (week 13). PROD activity was also significant and dose related at 75-300 mg/kg/day (weeks 1 and 4) and at 25-300 mg/kg/day (13 weeks); peak induction occurred after 1 week of treatment. In mice, significant induction of P450 was noted but only at 600 mg/kg/day for weeks 1, 4, and 13 and PROD induction was significant at 300 and 600 mg/kg/day at all sacrifice intervals. Additional investigations of hepatic microsomes were undertaken after 1 week of dosing with 150 or 300 mg/kg/day (rats) or 600 mg/kg/day (mice). As shown in Table 1 (of the article), regardless of the species, significant induction by the highest dose tested of microsomal protein (130 vs 110%, rats vs mice), 7-ethylresorufin O-deethylase, EROD (marker for CYP1A subfamily of isoenzymes) (415 vs 325 %, rats vs mice) and erythromycin N-demethylase (marker for CYP3A subfamily of isoenzymes) (155 vs 200 %, rats vs mice) was seen. Based on these findings, Western blot analysis with antibodies to rat CYP2B1/2 and CYP3A were preformed on microsomal fractions derived from mice treated with 0, 300 or 600 mg/kg *p*-DCB for 1week and rats similarly treated with 0, 75 and 300 mg/kg/day. As shown in Figure 5a (of the article), a dose-related increase in CYP2B1/2 was observed in *p*-DCB-treated mice and rats. As indicated, CYP2B1/2 expression was stronger in the rat. CYP3A expression was also induced in rats treated with 300 mg/kg/day (Figure 5b) but not in the mice. These results suggest that *p*-DCB is a CYP2B inducer in both species. The investigators concluded that this response is similar to phenobarbital. In support of this claim the investigators stated that like phenobarbital in rats and mice, p-DCB increased the liver weights, produced centrilobular hypertrophy, and stimulated replicative DNA synthesis after acute but not chronic exposure. They further cited the work of Grasso and Hinton (1991) indicating that mice are more

susceptible than the rat to liver tumor formation induced by nongenotoxic agents such as phenobarbital. To defend their position, the authors further stated, "...the data suggest that promotional and other effects of a phenobarbital-type enzyme inducer may be important in the formation of mouse liver tumors by DCB and may account for the observed species difference in hepatocarcinogenicity".

Figure 2 Dose and Temporal Response of Male Mice and Rats Treated with *p*-DCB : Liver Weights (Extracted from Lake et al., 1997)

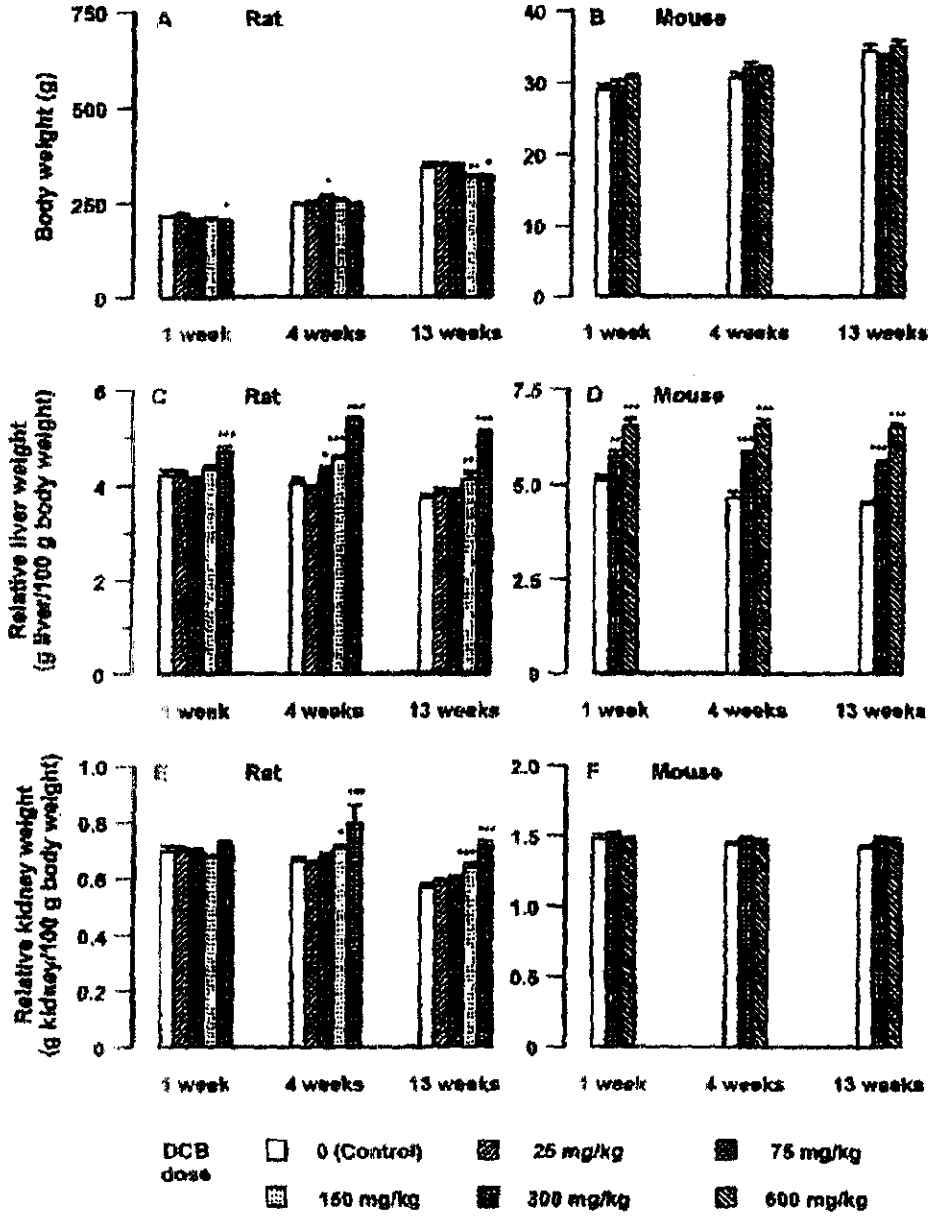


FIG. 1. Effect of treatment with 25–600 mg/kg DCB by daily oral gavage 5 days per week for 1, 4, and 13 weeks to rats (A, C, E) and mice (B, D, F) on body weight (A, B), relative liver weight (C, D), and relative kidney weight (E, F). Results are expressed as means ± SE of six to eight animals. Values significantly different from control (corn oil-treated animals) are: *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

Figure 3. Dose and Temporal Response of Male Mice and Rats Treated with *p*-DCB : BrdU

Labeling Index (Extracted from Lake et al., 1997)

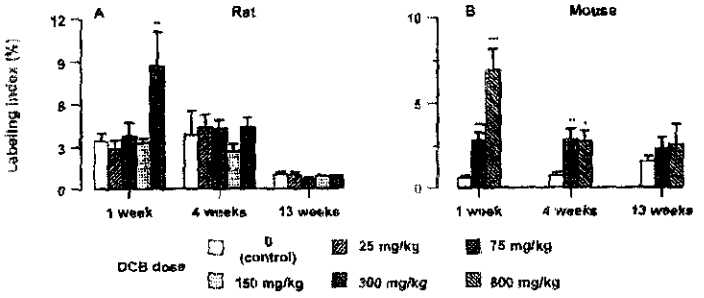


FIG. 2. Effect of treatment with 25–600 mg/kg DCB by daily oral gavage 5 days per week for 1, 4, and 13 weeks to rats (A) and mice (B) on the hepatocyte labeling index. Results are expressed as means ± SE of six to eight animals. Values significantly different from control (corn oil-treated animals) are: $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

Figure 4. Dose and Temporal Response of Male Mice and Rats Treated with *p*-DCB : Hepatic Microsomes (Extracted from Lake et al., 1997)

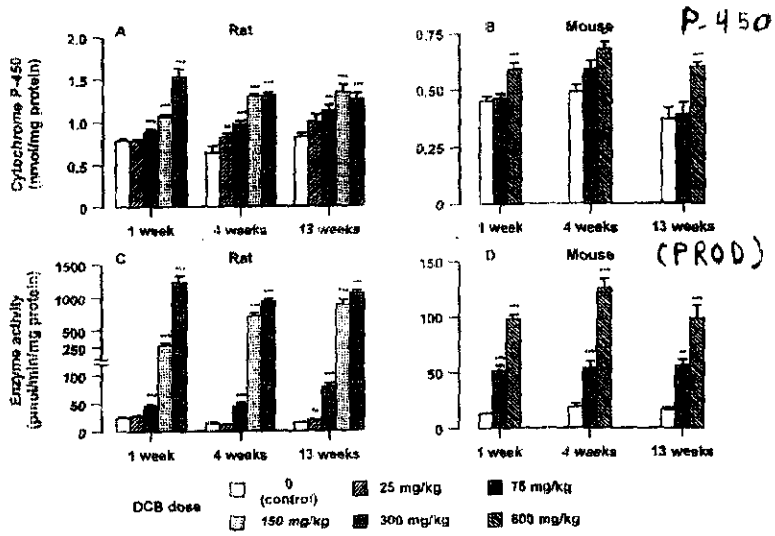


FIG. 4. Effect of treatment with 25–600 mg/kg DCB by daily oral gavage 5 days per week for 1, 4, and 13 weeks to rats (A, C) and mice (B, D) on hepatic microsomal cytochrome P450 content (*A, B*) and 7-*pentoxy*resorufin *O*-depentylase activity (C, D). Results are expressed as means ± SE of six to eight animals. Values significantly different from control (corn oil-treated animals) are: $^{**}p < 0.01$; $^{***}p < 0.001$.

Table 1. Response of Male Mice and Rats Treated with *p*-DCB : Hepatic Microsome Content (P-450) and Mixed Function Oxidase Activity (Extracted from Lake et al., 1997)

TABLE 1
Effect of DCB Treatment for 1 Week on Rat and Mouse Hepatic Microsomal Protein Content and Mixed Function Oxidase Activities

Parameter	DCB (mg/kg)				
	Rat			Mouse	
	Control	150	300	Control	600
Microsomal protein (mg/g liver)	21.2 ± 1.0[a]	24.7 ± 1.4	27.8 ± 1.0***	27.6 ± 0.8	30.5 ± 0.9*
7-Ethoxyresorufin *O*-deethylase (pmol/min/mg protein)	23 ± 2	63 ± 5***	95 ± 6***	13 ± 1	42 ± 5***
Erythromycin *N*-demethylase (nmol/min/mg protein)	0.78 ± 0.04	0.90 ± 0.05*	1.21 ± 0.05***	0.46 ± 0.07	0.93 ± 0.16*

Note. Values significantly different from control (corn oil-treated animals) are: *$p < 0.05$; ***$p < 0.001$.
[a] Results are expressed as means ± SE of six to eight animals.

Figure 5. Western Blot Analysis of Hepatic Microsomal Fractions from Male Mice and Rats Treated with *p*-DCB (Extracted from Lake et al., 1997)

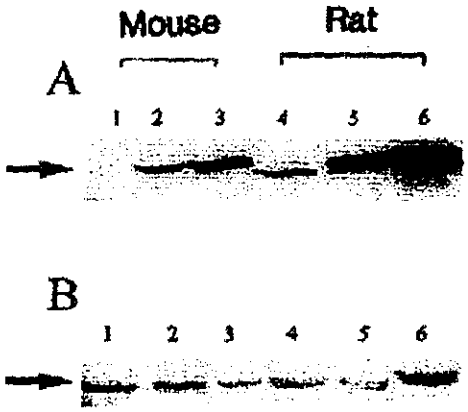


FIG. 5. Immunoblots of hepatic microsomal fractions incubated with antibodies to rat CYP2B1/2 (A) and rat CYP3A (B). Microsomal fractions (20 μg protein per lane) were prepared from pooled liver samples from mice treated with 0 (corn oil control), 300, and 600 mg/kg DCB (lanes 1, 2, and 3, respectively) and rats treated with 0 (corn oil control), 75, and 300 mg/kg DCB (lanes 4, 5, and 6, respectively) for 1 week.

In the oral study of Umemura et al., (1992), groups of five 6-week old B6C3F1 male mice were exposed to 0, 600, 1000 and 1800 mg/kg *p*-DCB (in corn oil) via intragastric (ig) administration. The high dose was selected based on preliminary data showing that no deaths occurred up to 7 days after treatment with 1800 mg/kg. The low dose was equivalent to the highest dose tested in the 1987 NTP study. Animals received a single intraperitoneal (ip) injection of 100 mg/kg BrdU 2 hours prior to sacrifice (2 days postdosing). Blood was measured

for alanine aminotransferase (ALT) activity and liver sections were harvested for immunohistochemical measurements of BrdU incorporation using the avidin-biotin-peroxidase complex (ABC) method. Liver sections from the left, right and anterior right lobe were subjected to histological examinations for quantification of necrotic areas (NA) that were defined as the percentage of the sum of the NAs divided by the total area examined in the three lobes. In addition a time-course study was conducted with groups of five mice dosed with 1800 mg/kg and sequentially sacrificed at 1,2,3,4, or 7 days posttreament. Animals in the time-course study were also examined for ALT activity, BrdU incorporation and quantification of NA.

Dose response study: As shown in Figure 6 (Figure 3 of the article), after a single administration of *p*-DCB, significant ($p<0.05$) increases in BrdU labeling were recorded at the two highest doses tested (1000 and 1800 mg/kg). At the high dose, a significant ($p<0.05$) increase in ALT activity was also demonstrated in the absence of a statistical significant but observable increase in NAs. The study authors noted that there were no significant changes in ALT or NAs at the lowest dose where BrdU activity was significantly increased (1000 mg/kg). This indicates, particularly, for the 1000-mg/kg-treatment group that cell proliferation occurs in the absence of overt liver toxicity.

Time-course study: Findings from the time-course study are illustrated in Figure 7 (Figure 4 of the article). As shown, administration of 1800 mg/kg *p*-DCB was associated with little or no ALT activity or liver necrosis while significant increases in BrdU labeling were noted at days 2 and 3 ($p<0.01$) and day 4 ($p<0.05$) as compared to the clear hepatotoxic effects induced by the ortho and meta isomers. Although the study authors stated that BrdU incorporation was not achieved at the oral tumorigenic dose in the mouse NTP study (600 mg/kg/day) only a single

Figure 6. Dose Response Study in Male Mice Treated with *p*-DCB: Liver Activity

272 UMEMURA ET AL.

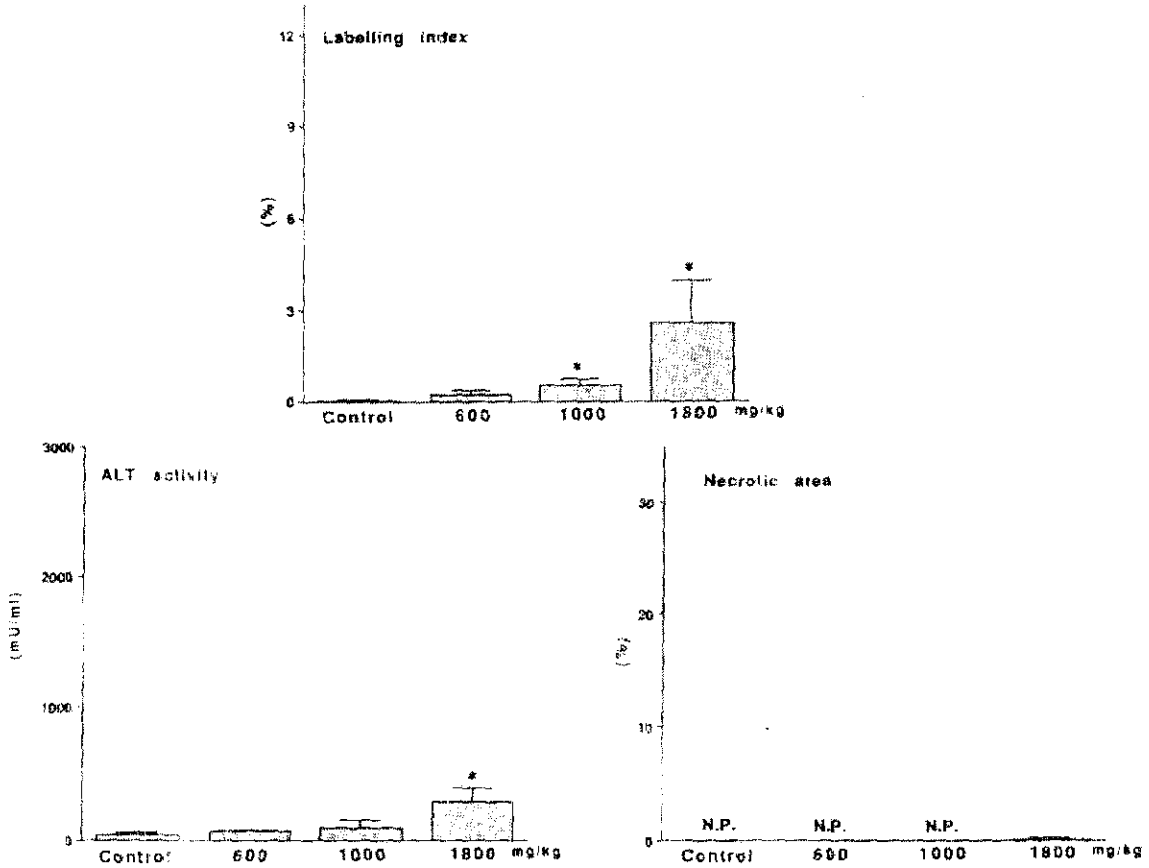
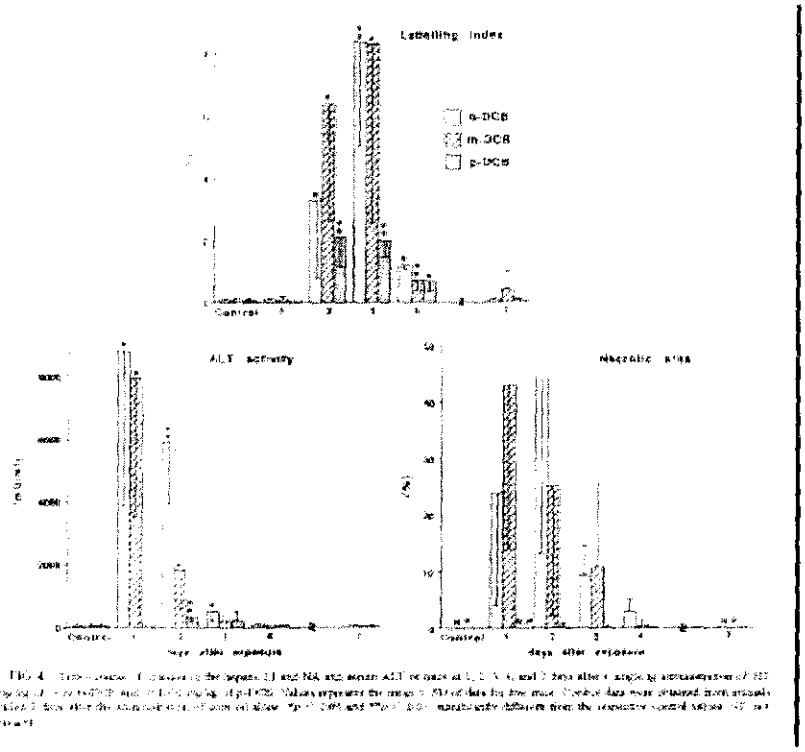


FIG. 3. Levels of hepatic LI and NA and of serum ALT in mice 2 days after a single ig administration of *p*-DCB at doses of 600, 1000, or 1800 mg/kg. Values represent the mean ± SD of data for five mice. *$p < 0.05$: significantly different from the respective control values. NP, not present.

Extracted from Umemura et al, 1992

Figure 7. Time Course Study in Male Mice Treated with *p*-DCB: Labeling Index, ALT Activity and Necrotic Areas of Mice at 1,2,3,4 and 7 days after single ig administration of 1800 mg/kg



Extracted from Umemura et al, 1992

administration of this level was studied. Nevertheless, the finding of cell proliferation in the absence of overt hepatotoxicity is consistent with the results of Eldridge et al (1992) (discussed below) who also found evidence of mitogenic stimulation.

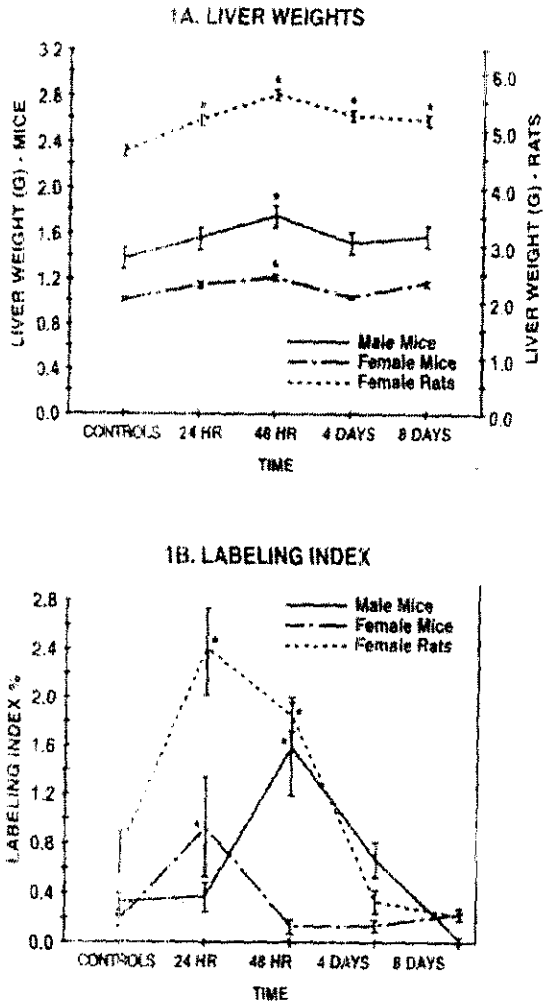
In this study, Eldridge and coworkers performed a series of cell proliferation assays "under conditions of the NTP bioassay". Accordingly, a **time-course single dose** study was conducted with groups of five 10-week old B6C3F1 male and female mice and female F344 rats receiving oral gavage doses of 600 mg/kg p-DCB (in corn oil). At days 1, 2, 4, and 8 posttreatment, all animals were injected i.p. with 20 mg/kg BrdU, and sacrificed after 2 hours. For the **dose-response analysis**, additional mice (5/sex) and rats (5♀) were administered oral gavage doses of 0, 600, 900 or 1200 mg/kg and sacrificed 24 or 48 hours after dosing. A 13-week **time-course** study was also conducted with male and female mice and female rats oral gavaged with 0, 300 or 600 mg/kg/day (mice ♂♀) or with 0 or 600 mg/kg/day (♀ rats) 5 days/week for 13 weeks. On week 5, the treatment was discontinued for high-dose male and female mice and female rats; this subgroup constituted the **"STOP" phase of the experiment** and received corn oil in place of the p-DCB for an additionally week. BrdU pumps were implanted mid-week 6 and sacrifices were performed at the end of week 6. The remaining animals were sequentially implanted BrdU pumps at weeks 1, 3, 6 and 13 and sacrificed after 3 days. In all assays, liver enzyme levels

(AST, ALT, LDH and SDH (sorbitol dehydrogenese) and liver weights were determined; labeling indices (LIs) were ascertained for all animals except those in the dose-response study. Liver histopathological examinations were done on all animals but those in the single dose time-course investigation.

Results from the **single dose time-course experiment** are presented in Figure 8 [Figures 1A (liver weights) , Figure 1B (Labeling Index (LI)) in the article] and Figure 9 [Figure 2 (ALT activity) in the article]. As shown, liver weights of mice and rats were significantly ($p<0.05$) increased at all timed intervals (1 to 8 days). LIs were also significantly increased for all animal groups at 24 and/or 48 hours but not at the later timed intervals as follows: male rats, 48 hrs, female mice, 24 hrs and female rats, 24 and 48 hours. No appreciable increases in AST, ALT, LDH or SDH were evident after 1, 2, 4 or 8 days posttreatment. The authors stated that these findings indicate a lack of overt hepatocellular injury. Findings from the **dose-response study** are in agreement with these results and indicate that liver enzyme values were generally comparable to the vehicle controls for all treatment groups of male or female mice and female rats up to a level (1200 mg/kg) that exceeded the tumorigenic dose (600 mg/kg) in mice. The lack of a toxic effect on the liver supports the histological results reported by the investigators which indicate that only vacuolation and basophilic granules were seen in the periportal and/or centrilobular hepatocytes of male, and to a lesser extent in female mice but no necrosis in either sex of mice or female rats.

Data from the "**STOP" phase of the 13-week time-course experiment** are illustrated in Figure 10 3 A-C (liver weight) and 4 A-C (LIs) from the article. These data show that liver weights were significantly ($p<0.05$) increased at 600 mg/kg/day in male and female mice and female rats for weeks 1-13. At the lowest assayed dose (300 mg/kg/day), significant increases were achieved for male and female mice at weeks 6 and 13. In the **"STOP EXPERIMENT"**, liver weights of mice (♂♀) returned to the control values after 1 week of cessation of treatment with 600 mg/kg/day for 5 weeks while rat livers decreased in weight, they, nevertheless, remained significantly higher than control. As further presented, the LIs for male and female mice were significantly ($p<0.05$) increased at 600 mg/kg/day but not at 300 mg/kg/day. Although the peak of the response was recorded after 1 week of treatment, a significant increase was still apparent for male mice at week 3. The investigators reported that heavily labeled hepatocytes were most frequently observed in the centrilobular region of male mice livers while labeled hepatocytes were distributed throughout all lobes in the female mice. Significantly ($p<0.05$) increased LIs were also seen in female rats at 600 mg/kg/day; this response peaked at week 1 posttreatment and no other significant increases were observed. In the **"STOP EXPERIMENT"**, LI were lower than control 1 week after removal of treatment for both species. No adverse effects on liver enzyme levels or evidence of liver necrosis were observed after 13 weeks of treatment with *p*-DCB. The only differences noted in the histological examination after 13 weeks of treatment was hypertrophy of centrilobular hepatocytes with enlarged hyperchromic nuclei in male and female mice of the 600-mg/kg/day test group; alterations of hepatocytes were not observed in the mice treated with 300 mg/kg/day or in the female rats treated with 600 mg/kg/day.

Figure 8: Figures 1A and 1B. Liver Weights and Labeling Indices from Male and Female B6C3F1 Mice and Female F344 Rats Following a Single Oral Exposure to *p*-DCB Figures were extracted from Eldridge et al., 1992.



Figures 1A and 1B. Liver weights (A) and hepatocellular Labeling Index [LI] (B) for male and female mice and female rats given a single administration of DCB in corn oil by gavage at the highest NTP bioassay dose (600 mg/kg). Animals were killed following treatment at the times indicated. (A) Liver weights of control and treated animals. (B) LI of control and treated animals. BrdU was administered via i.p. injection 2 hr. prior to killing of the animals.

Figure 9. ALT Activity from Male and Female B6C3F1 Mice and Female F344 Rats Following a Single Oral Exposure to *p*-DCB

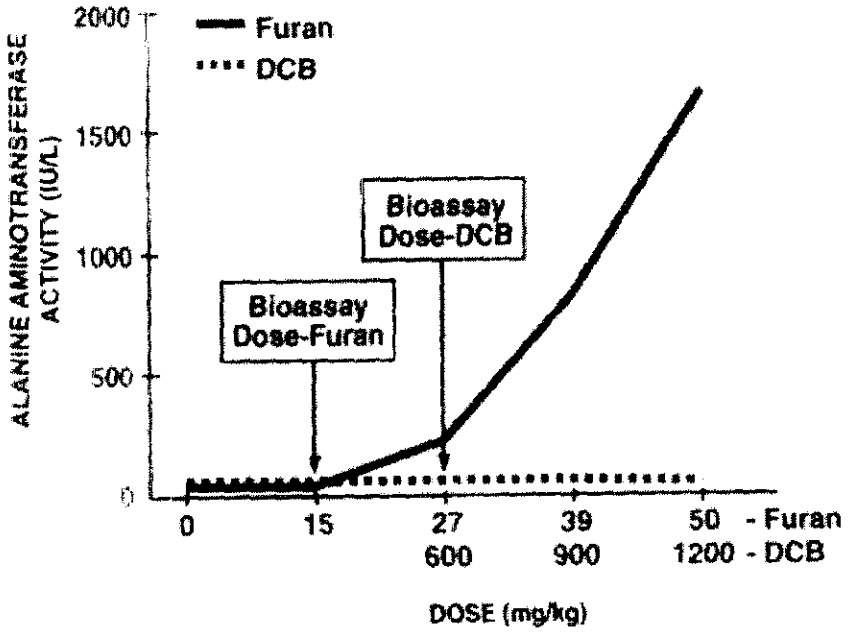
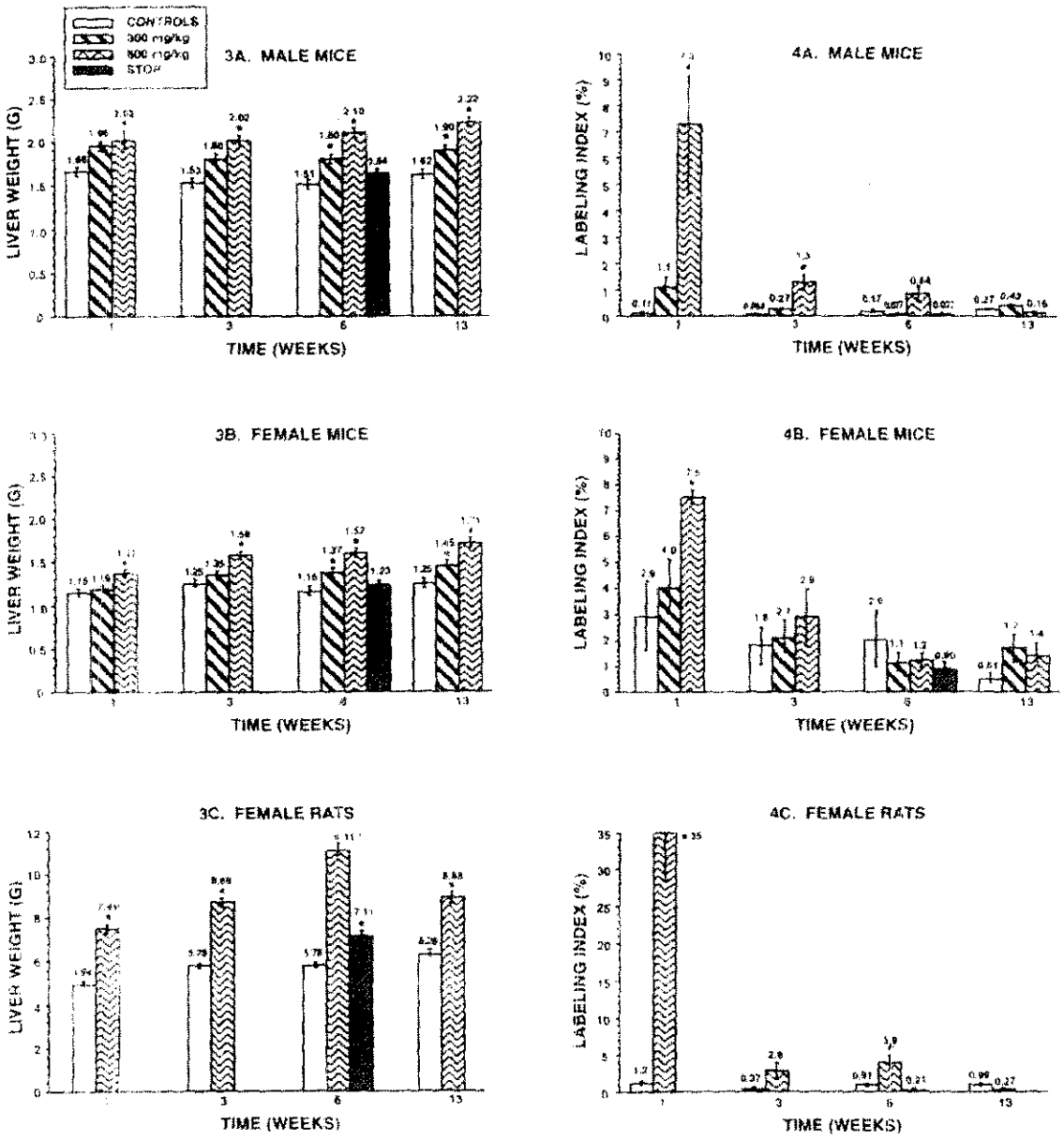


Figure 2. Activity of ALT in the plasma of male mice receiving a single gavage administration of furan (cytotoxicant) or DCB (mitogen) in corn oil at the doses indicated. Animals were killed 24 h. following treatment. Each data point represents the mean of five animals. Only animals treated with furan at doses greater than the bioassay dose differed significantly from controls.

Figure was extracted from Eldridge et al., 1992

Figure 10: Figures 3A-C and 4A-C Liver Weights and Labeling Indices from Male and Female B6C3F1 Mice and Female F344 Rats Orally Exposed to *p*-DCB for 13 weeks



Figures were extracted from Eldridge et al., 1992

From the overall results, the investigators concluded that *p*-DCB induces a dose-related mitogenic stimulation of cell proliferation (rather than a regenerative response to hepatotoxicity) in the livers of male and female mice, which parallels the oral dose-related tumor response in mice. This conclusion is supported by the evidence of increased liver weights and increased LIs without observable liver damage or increases in liver enzyme activities. In addition, the reversibility of the liver response when mice were withdrawn from treatment supports this position. This activity occurred at the oral tumorigenic dose for mice (600 mg/kg/day). However, the same proliferative response was observed in F344 female rats in the absence of a tumorigenic response. As noted earlier, the only difference between the species was centrilobular liver hypertrophy in male and female mice but not in female rats. The investigators speculate, therefore, that species differences in metabolism of *p*-DCB and/or the refractory nature of liver tumors in rats vs. mice account for the susceptibility of the mouse liver and the resistance of F344 rat liver to *p*-DCB- induced liver tumors. For the latter possibility, the investigators cited the high and variable spontaneous hepatocellular carcinoma incidence rate for B6F3C1 male mice as 7-55% and for female mice as 2-8% (Doull et al., 1983) versus the much lower spontaneous rate for male and female F344 rats (0.8 and 0.2%, respectively as cited in Haseman et al., 1990). This line of reasoning is in agreement with Butterworth (2006) who states, "B6C3F1 mice are genetically predisposed and extremely susceptible to induced liver cancer".

Inhalation Studies

In the subchronic inhalation study of Aiso et al. (2005b), BDF_1 mice and F344 rats of both sexes were exposed to atmospheres containing 25, 55, 120, 270 or 600 ppm p-DCB 6 hours/day, 5 days/week for 13 weeks. Liver weights, liver enzyme activity and histopathology findings for mice are summarized in Table 2. As shown, absolute liver weights at 600 ppm (♂) and 270 and 600 ppm (♀) were significantly increased (↑ in males: 49% and in females: 43% and 11%, respectively). Relative (to body weight) liver weights were also significantly increased at all concentrations (25-600 ppm) in the males and at 270 and 600 ppm in females. These increases for the males were 61% at 600 ppm, 22% for 270 ppm and 8% for 120 ppm compared to the air control and 33% for high dose females. These effects on organ weight were accompanied by significant increases in AST and ALT at 600 ppm (♂: 55 and 64%, respectively) and ALT (♀: 100%) only at 600 ppm in females. Nevertheless, the incidence of animals with focal necrosis was low and seen only in two male mice (1 slight, 1 moderate) at 600 ppm. In contrast, 10 of 10 males and 10 of 10 females in the highest treatment group and 10/10 males at 270 ppm had significantly ($p \leq 0.01$) increased hepatocellular hypertrophy. In agreement with these data are the findings from the 2-year bioassay also performed by Aiso et al. (2005a) (see Tables 3 and 4 in Cancer Assessment Document) showing significantly increased absolute and relative liver weights in both sexes and hepatocellular hypertrophy in 34 of the 49 male mice (69%; 25 with slight and 9 with moderate grades of severity) exposed to an atmosphere of 300 ppm of p-DCB in the 2-year bioassay. However, only 2 of 50 female mice (4%) exhibited hepatocellular hypertrophy (1 had slight and the other had a moderate grade of hypertrophy).

Table 2. Select Liver Weights, Biochemical Determinations and Histological Findings of Male and Female BDF1 Mice exposed to p-DCB vapors for 13 weeks[a]

Males[b]

	0	120 ppm[c]	270 ppm	600 ppm
Body Weight (g)	31.2±3.7	29.6±2.3	28.1±3.1	28.2±3.4
Absolute Liver (g)	1.099±0.09	1.155±0.08 (5%)	1.233±0.12 (12%)	1.633**±0.26 (49%)
Relative (%)	3.6	3.9* (8%)	4.4** (22%)	5.8** (61%)
AST (IU/l)	42±5	46±13 (10%)	50±10 (19%)	65±19** (55%)
ALT (IU/l)	11±2	13±4	17±3 (55%)	29±6** (64%)
Hepatocellular Hypertrophy: Centrilobular	0	0	10** (1+: 10)[d]	10** (2+: 10)[d]
Necrosis: Focal	0	0	0	2 (1+:1; 2+:1)[d]

Females[b]

	0	120 ppm[c]	270 ppm	600 ppm
Body Weight (g)	20.9±0.6	21.7±1.8	21.4±1.2	22.2±1.1
Absolute Liver (g)	0.890±0.04	0.954±0.08 (7%)	0.984*±0.09 (11%)	1.272**±0.08 (43%)
Relative (%)	4.3	4.4	4.6** (7%)	5.7** (33%)
AST (IU/l)	58±13	60±24	65±20 (12%)	75±13 (29%)
ALT (IU/l)	14±3	14±6	15±2	28±5** (100)
Hepatocellular Hypertrophy: Centrilobular	0	0	0	10* (1+: 1; 2+: 9)[d]
Necrosis: Focal	0	0	0	0

[a] Extracted from Aiso et al (2005b), Tables 1, 2, and 4.
[b] 10 /sex/group: means and standard deviations
[c] In general, no appreciable increases in the above parameters were seen at lower concentrations (25 or 55 ppm)
[d] Severity grades: 1+ = slight, 2+ = moderate, 3+ = marked, 4+= severe
* Significant increase at $p \leq 0.05$ by Dunnett's test
**Significant increase at $p \leq 0.01$ by Dunnett's test.

Liver weight, liver enzyme activity and histopathology for F344 rats in the 13-week inhalation study are presented in Table 3. As shown, the liver weight response in rats generally followed the response observed in the B6C3F1 mice (i.e., S↑ absolute and relative liver weights at 600 ppm but also at lower doses in rats). Nine of 10 male rats had slight hepatocellular hypertrophy at the high dose only as compared to 100% of the male mice at both 270 and 600 ppm.

Similarly, three high-dose female rats had slight hepatocellular hypertrophy while 100 % of the female mice at a comparable concentration generally exhibited moderate liver hypertrophy. This difference continues through the 2-year inhalation bioassay where 34 of 49 male mice presented with hepatocellular hypertrophy and only 5 of 50 male rats showed this finding. Hence, there is support for the conjecture of Eldridge et al, (1992) that mouse livers are more susceptible and F344 rat liver are more resistant to p-DCB induced liver tumors, bearing in mind the historical spontaneous frequency of liver tumors in male B6C3F1 as reported by Doull et al. (1983): B6F3C1 male mice = 7-55% and for female mice as 2-8% (Doull et al., 1983) versus the much lower spontaneous rate for male and female F344 rats as 0.8 and 0.2%, respectively (Haseman et al., 1990).

Summary of Oral and Inhalation Mode of Action Studies

In summary, Tables 4 through 5 depict the similarities in the response of both rodent species and both sexes regardless of the route of exposure to treatment with p-DCB. All animals showed increased liver weights (generally detectable 48 hours after treatment and continuing until study termination at 2 years) and increased LIs (Tables 4), generally starting after 24-48 hours (105- to 150-fold in male mice at 1800 mg/kg or 4-fold at 600 mg/kg) but declining after 4 days (to 2.5-fold at 600 mg/kg in male mice). A similar pattern was seen in the female mice and female rats. Proliferative activity was generally not accompanied by appreciable liver toxicity at any time during the course of the various studies. The only differences are in the tumor response and the degree of liver hypertrophy in both the oral gavage and inhalation studies of p-DCB. For example, liver hypertrophy was first seen in the 13 week subchronic oral study and male and female mice orally administered 600 mg/kg/day for 2 years had an incidence of 80 and 54% liver hypertrophy; this finding was not seen in the rats. In the inhalation studies, 100% of male mice at 270 or 600 ppm and 100% of female mice at 600 ppm presented with liver hypertrophy after 13 weeks of treatment, and 69% of the males and 4% of the females showed hypertrophy after 2 years of treatment.

Table 3. Select Liver Weights, Biochemical Determinations and Histological Findings of Male and Female F344 Rats exposed to p-DCB vapors for 13 weeks[a]

Males[b]

	0	120 ppm[c]	270 ppm	600 ppm
Body Weight (g)	318±14	327±13	334±19	321±12
Absolute Liver (g)	7.751±0.48	8.522*±0.38 (10)	9.402**±0.76 (21%)	11.23**±1.01 (45%)
Relative (%)	2.4	2.6* (8%)	2.8** (17%)	3.5** (46%)
AST (IU/l)	74±7	75±15	69±11 (7%↓)	54±3** (27%↓)
ALT (IU/l)	25±2	24±3	21±3** (16%↓)	16±2** (36%↓)
Hepatocellular Hypertrophy: Centrilobular	0	0	3 (1+: 3)[d]	9** (1+: 9)[d]
Necrosis: Focal	0	0	0	0

Females[b]

	0	120 ppm[c]	270 ppm	600 ppm
Body Weight (g)	175±10	176±8	178±7	182±8
Absolute Liver (g)	4.031±0.23	4.128±0.20 (2%)	4.392*±0.19 (9%)	5.349**±0.27 (33%)
Relative (%)	2.3	2.3	2.5** (9%)	2.9** (26%)
AST (IU/l)	78±26	77±20	72±14 (8%↓)	56**±4 (28%↓)
ALT (IU/l)	26±15	28±15	23±6	17±3
Hepatocellular Hypertrophy: Centrilobular	0	0	0	3 (1+: 3)[d]
Necrosis: Focal	0	0	0	0

[a] Extracted from Aiso et al (2005b), Tables 1, 2, and 4.

[b] 10 /sex/group, means and standard deviations

[c] In general, no appreciable increases in the above parameters were seen at lower concentrations (25 or 55 ppm)

[d] Severity grades: 1+ = slight, 2+ = moderate, 3+ = marked, 4+ = severe

* Significant increase at p≤ 0.05 by Dunnett's test

**Significant increase at p≤ 0.01 by Dunnett's test.

Table 4. Comparative Analysis of Key Factors Influencing Liver Tumor Formation in B6C3F1 Mice but not in F-344 Rats Expose Orally to p-DCB[a]

Key Factors	Male Mice	Male Rats	Female Mice	Female Rats
P-450 Induction	+; detectable 1 & 13 wk at 600 mg/kg/day	Not done	+; detectable 1 & 4 wk at 75, 150 & 300 mg/kg/day & 13 wk at 75, 150 & 300 mg/kg/day	Not done
Cell Proliferation (BrdU Labeling Index, LI)	+; detectable 48 hrs; 1, 2 & 4 wk at 300 and 600 mg/kg	+ wk 1 but not wk 4 at 300 mg/kg	+;detectable 24 hrs; 1 wk at 300 and 600 mg/kg	+;detectable 24 & 48 hrs; 1wk at 300 mg/kg
Hepatocellular Hypertrophy	+ after 13 wks at 300 and 600 mg/kg; 80% at 600 mg/kg day after 2 yrs	NONE after 13 wks or 2 yrs	+ after 13 wks at 600 mg/kg/day; 54% at 600 mg/kg/day after 2 yrs	NONE after 13 wks or 2 yrs
Increased Liver Weights	+; detectable 48 hrs still present after 2 yrs at 300 and 600 mg/kg/day	+; detectable 48 hrs still present after 2 yrs at 150 and 300 mg/kg/day	+; detectable 48 hrs still present after 2 yrs at 300 and 600 mg/kg/day	+; detectable 48 hrs still present after 2 yrs at 150 & 300 mg/kg/day
Stop/Recovery	Liver wts % LIs return to normal 1 wk post-dosing at 600 mg/kg/day	Not Done	Liver wts % LIs return to normal 1 wk post-dosing at 600 mg/kg/day	Liver wts ↓ but still S; LIs return to normal 1 wk post-dosing at 300 mg/kg/day
Liver Necrosis or ↑Enzyme Activity Showing Liver Injury[b]	NONE	NONE	NONE	NONE
Liver Tumors	S↑ Adenomas & Carcinomas at 600 mg/kg/day	No S↑	S↑ Adenomas & Carcinomas at 600 mg/kg/day	No S↑

[a] Data were derived from the oral studies of Umemura et al. (1992), Eldridge et al. (1992), and the NTP 2-year study (1987). [b] ALT, AST, LDH, and/or SDH

Table 5. Comparative Analysis of Key Factors Influencing Liver Tumor Formation in BDF1 Mice but not in F-344 Rats Expose via Inhalation to p-DCB[a]

Key Factors	Male Mice	Male Rats	Female Mice	Female Rats
Cell Proliferation (BrdU Labeling Index, LI)	Not Done	Not Done	Not Done	Not Done
Hepatocellular Hypertrophy	10 of 10 + after 13 wks at 270 & and 600 ppm; 69% at 300 ppm after 2 yrs (moderate severity)	9 of 10 + after 13 wks at 600 & 3 of 10 at 270 ppm; 10% at 300 ppm after 2 yrs (slight severity)	10 of 10 + after 13 wks at 600 ppm; 4% at 300 ppm after 2 yrs (slight to moderate severity)	3 of 10 after 13 wks at 600 ppm; 6% at 300 ppm after 2 yrs (slight severity)
Increased Liver Weights	+ after 13 weeks at 270 & 600 ppm still present after 2 yrs at 300 ppm	+ after 13 weeks at 120, 270 & 600 ppm still present after 2 yrs at 300 ppm	+ after 13 weeks at 270 & 600 ppm still present after 2 yrs at 300 ppm	+ after 13 weeks at 270 & 600 ppm still present after 2 yrs at 300 ppm
Stop/Recovery	Not Done	Not Done	Not Done	Not Done
Liver Necrosis or ↑ Enzyme Activity Showing Liver Injury[b]	S↑ liver enzymes; 20% w focal necrosis after 13 wks at 600 ppm	NONE	S↑ liver enzymes; 0% w focal necrosis after 13 wks at 600 ppm	NONE
Liver Tumors	S↑ Adenomas & Carcinomas at 300 ppm	No S↑	S↑ Adenomas & Carcinomas at 300 ppm	No S↑

[a] Data were derived from the inhalation studies of Aiso et al. (2005a,b).

[b] ALT, AST, LDH, and/or SDH

Alternative Modes of Action

1. Hepatotoxicity: Aiso et al. (2005a) concluded that hepatocellular injury and regenerative cell proliferation as well as genotoxicity were involved in the p-DCB-induced hepatocarcinogenicity in mice. As shown in Table 2 from the 13-week subchronic inhalation study performed by the same author (Aiso et al., 2005b), however, hepatocellular hypertrophy occurred in 100% of the male and female mice exposed to 600 ppm in the absence of clear evidence of hepatotoxicity (i.e., only 20% of the males and no females displayed focal necrosis). Similarly, 69% of the males and 4% of the females had increased hepatocellular hypertrophy in the absence of histolopathologic changes indicating hepatotoxicity in either sex in the 2-year bioassay. Although by a different exposure route (ig), no increases in necrotic areas of the liver were seen in male mice exhibiting significantly increased BrdU labeling after a single exposure to 1800 and 600 ppm p-DCB (Figure 7). Similarly, the findings from the oral gavage series of cell proliferation assays performed by Eldridge et al. (1992) provide clear evidence of increased liver weights and increased LIs without observable liver damage or increases in

liver enzyme activities at 1200 mg/kg (Figures 8A- 11A-C), a dose that exceeds the tumorigenic dose of 600 mg/kg/day in mice. This evidence argues against hepatocellular injury and regenerative cell proliferation as the MOA for the p-DCB-induced liver tumors.

2. Peroxisome Proliferation: No data were found in the open literature evaluating the effects on palmitoyl-CoA oxidation or changes in the size or number of peroxisomes.

3. Mutagenicity: Refer to Section IV.2 in the Cancer Assessment document.

Relevance to Humans

The key events presented for this non-genotoxic MOA are plausible in humans. It is likely that the downstream events of increased cell proliferation and the tumor induction would ensue if a dose were reached that produced similar liver perturbations. The MOA is applicable to all populations, including children. Although metabolic enzyme systems in children do not reach adult levels of activity until 6 months to 1 year of age, we should, nevertheless, assume that this MOA is operational in general.

Conclusions:

Based on the overall analysis, we conclude that a plausible non-genotoxic MOA involving mitogenesis was established for the p-DCB-induced liver tumors in male and female mice. This conclusion is based on the following evidence:

1. *p*-DCB is not mutagenic. With the exception of single assays showing DNA damage *in vitro* and *in vivo*, data from genetic toxicology studies are negative. These positive results were seen, however, in the absence of a clear mutagenic effect (i.e., gene mutations or chromosome aberrations). Consequently, data are not sufficient to further consider a mutagenic MOA for p-DCB.
2. There is dose-concordance between liver tumors, hepatic microsomal enzyme induction and cell proliferation in mice.
3. Cell proliferation occurred in male and female mice in the absence of overt liver toxicity
4. Temporal relationship supporting this MOA was demonstrated. The mitogenenic proliferative response was identified at the tumorigenic dose as early as 1 day (females) or 2 days (males) after the onset of treatment. The response declined after 4 days.

The characteristics of the response were also demonstrated in female and/or male rats in the absence of tumor formation. As stated earlier, the only major difference between mice and rats was the intensity of the effect with respect to centrilobular liver hypertrophy. However, several authors (Grasso and Hinton, 1991; Eldridge et al., 1992; Haseman et al., 1990) have observed similar findings and have concluded that mice, particularly the B6C3F1strain, are more susceptible to liver tumors than rats. Additionally, *p*-DCB is more extensively metabolized by the B6C3F1 mouse than the F-344 rat.

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